THE FABULOUS 3: AN EXPLORATION OF ADIPOSE DERIVED STEM CELLS, NEUROTROPHIC FACTORS AND FIBRIN HYDROGEL FOR PERIPHERAL NERVE REPAIR AND REGENERATION

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TABLE OF CONTENTS

SUMMARY

Background: Injuries to the peripheral nerve system are a common clinical problem which affect mostly young individuals. Standard treatment methods are well established, but are associated with several undeniable disadvantages that may have a long-lasting impact on the patients' quality of life. Therefore, peripheral nerve regeneration and repair is an active field of research with a variety of treatment strategies. However, most of them fail to be translated into clinical practice. **Objective:** The aim of the present thesis was to investigate translational approaches for enhancing peripheral nerve regeneration as well as for the surgical nerve repair. **Methods:** The first manuscript of this thesis focused on the neurotrophic potency of adipose derived stem cells (ASC) in response to growth factors stimuli, i.e. NGF or VEGF *in vitro* and *in vivo*, and the impact of two different stem cell delivery strategies, i.e. intramural versus intraluminal loading, in fibrin nerve conduits (FNC) on early nerve regeneration. The second manuscript evaluated the neurotrophic effect of ASC after stimulation by a variety of neurotrophic factors (NTF), i.e., NGF, BDNF, NT3, NT4, GDNF and CNTF, and investigated the neuroregenerative events taking place in ASC and sensory neuronal cells upon stimulation. In the third manuscript, we conducted a *prospective, randomized, two-arm-controlled phase-1 clinical trial* comparing the outcome of digital nerve injuries with or without nerve gap after experimental treatment with the fibrin nerve conduit (FNC) or the epineural suture with fibrin sealant versus the standard treatment with the autologous nerve graft or the epineural suture over a period of 12 months. **Results:** NTF-stimulated ASC promoted the axonal regeneration *in vitro* and *in vivo*, with NT3-stimulation revealing a specifically significant effect. Analysis of regenerative events taking place in ASC and sensory neuronal cells upon NT3-signaling resulted in up-regulation of regeneration associated genes and changing phosphorylation sites in both cell types. FNC based intramural delivery of NGF-stimulated ASC promoted better axonal regeneration *in vivo* than intraluminal delivery. In the clinical context, the FNC and the fibrin sealant may enhance the early nerve regeneration and performed non-inferior to standard treatments for peripheral nerve repair. **Conclusion:** Ex-vivo stimulation of ASC by NTF and FNC assisted intramural delivery may offer new options for the development of effective stem cell-based therapies, while the FNC and the fibrin sealant might already present viable clinical treatment alternatives for the repair of short-gap nerve injuries.

LIST OF ABBREVIATIONS

Abbreviation	Full term
ASC	Adipose derived stem cell(s) or unstimulated ASC
FNC	Fibrin nerve conduit(s)
NC	Nerve conduit(s)
NTF	Neurotrophic factor(s)
NGF	Nerve growth factor
BDNF	Brain-derived neurotrophic factor
NT3	Neurotrophin 3
NT4	Neurotrophin 4
GDNF	Glial cell line-derived neurotrophic factor
CNTF	Ciliary neurotrophic factor
SC	Schwann cell(s)
MSC	Mesenchymal stem cell(s)
BMSC	Bone marrow derived stem cell(s)
SCLC	Schwann cell-like cell
CM	Conditioned medium/a
DRG	Dorsal root ganglion(s)
STAT-3	Signal transducer and activator of transcription 3
GAP-43	Growth Associated Protein 43
SCLC	SC-like cells
GM	Growth medium
NTF-ASC	NTF-stimulated ASC
CM-NTF-ASC	CM from NTF-stimulated ASC
CM-ASC	CM from unstimulated ASC
RAG	regeneration associated gene(s)
FRMD8	FERM domain-containing protein 8
NOTCH2	Neurogenic locus notch homolog protein 2
TBCEL	Tubulin Folding Cofactor E Like protein
EVL	Ena/Vasp-Like protein
MAP1A	Microtubule-associated protein 1A

INTRODUCTION

1. ANATOMY OF THE PERIPHERAL NERVE

Peripheral nerves origin from the dorsal and the ventral roots which emerge from the spinal cord: the ventral root extends from motor neurons whose cell bodies are found in the ventral horn of the spinal cord. The dorsal root comes from sensory neurons whose cell bodies lie in the dorsal root ganglions. Peripheral nerves are the connecting structure between the central nervous system and distal sensory and motor target organs. The smallest functional unit of a nerve is the axon, a long cytoplasmic extension coming from the neuronal body. A connective tissue layer, the endoneurium, surrounds each axon. Multiple axons are arranged to spatially distinct bundles called fascicles, which are separated by the perineurium. Groups of fascicles are surrounded by the inner and outer epineurium, which define the nerve anatomically. A delicate network of capillaries exists at the level of the endoneurium. External to the epineurium is the mesoneurium, which contains the major blood supply for the nerve [1–3].

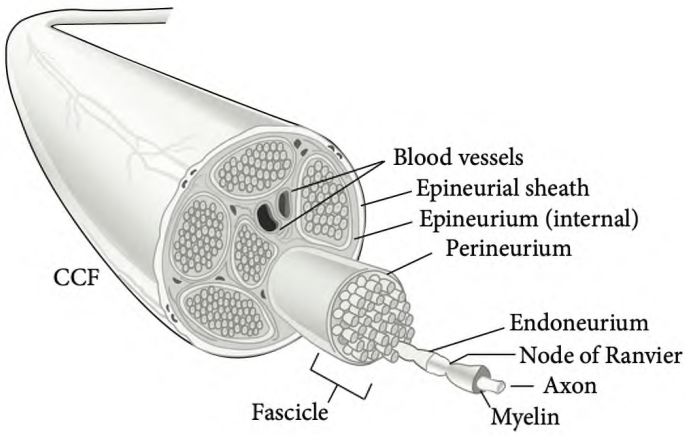

Figure 1. Anatomy of the peripheral nerve. *Figure from Siemionow and Brzezicki et al. [3]*

2. PERIPHERAL NERVE INJURY, DEGENERATION AND REGENERATION - AN OVERVIEW

2.1. Etiology and Classification of Peripheral Nerve Injuries

Injuries to peripheral nerves are a common clinical problem which experience increasing incidence. Typical patients affected are young males with a mean age around 30 years [4–6]. Most of the injuries are caused by motor vehicle, domestic or work related accidents [4,5,7,8] due to transection, compression, traction and laceration mechanisms [7,9]. Despite significant advances in microsurgical techniques and the patient-centered follow-up, the functional outcome after peripheral nerve repair may remain suboptimal, resulting in life-long sensory-motor deficits and in some cases pain [1,10,11]. Patients therefore experience

a profound and sometimes permanent impact on their quality of life, and the economic implications for both, the patient and the society are considerable [4,5,12,13].

For the classification of nerve lesions three main groups were defined by Sir Herbert Seddon in 1942 [14]: neurapraxia, axonotmesis and neurotmesis. Neurapraxia is a transient condition, resulting from traction or compression of the nerve. It leaves a physically intact nerve, but the mechanical stress acting on the conductive structures may still cause impairment of motor and sensory functions [7]. With the correct treatment, it regenerates spontaneously. In axonotmesis, the axons and the myelin sheath are damaged, but the endoneurium, perineurium and epineurium remain intact. Thus, also here spontaneous healing is possible [15]. However, neurotmesis is a complete transection of the nerve with its ensheathing structures, which is unlikely to recover without surgical repair [1,16]. Sunderland refined this classification further in 1951 [17], suggesting five degrees of severity, verified by using histological analyses.

2.2. Peripheral Nerve Degeneration and Regeneration

In contrast to the central nerve system where neuronal regeneration is restricted due to an unfavorable environment [9,18,19], the adult peripheral nerve system displays the astonishing ability to support neuronal survival and axonal regeneration after nerve injury. The regeneration capacity relates to 1) the patient's age with youth being a positive predictor, 2) the surgeon's experience, 3) the mechanism and severity of the injury, 4) the timing or delay of nerve repair, 5) the gap length of the nerve defect, 6) the anatomical site of the injury with distal nerve injuries recovering better than proximal nerve injuries and 7) adequate medical treatment and follow-up period [10,20]. Severed nerve fibers will degenerate within 24 to 48 hours after the injury [16]. In case of axotomy, the distal nerve end undergoes a process called Wallerian degeneration, which was first described by Augustus Waller in 1850 [9]. Calcium influx into the injured axons activates axonal proteases, which degenerate the axoplasm and axolemma [9]. Schwann cells (SC) dedifferentiate, switching from myelinating or non-myelinating cells to a regenerative phenotype [21]. These SC proliferate, initiate the myelin sheath breakdown, induce an inflammatory response for wound healing, and secrete neurotrophic factors, cell adhesion molecules and chemokines [21]. Macrophages enter the injury site [9] and, along with SC, contribute to debris clearance for a period of 3 to 6 weeks [7]. Meanwhile, also the proximal nerve end degenerates until

the first node of Ranvier [16]. Retrograde signaling along the proximal nerve end initiates metabolic modifications in the perikaryon, which prepares for physiological regeneration [9]. Regeneration associated pathways are activated in the neuronal body, which upregulate the transcription of structural proteins such as beta-tubulin 3 and actin. These proteins are transported to the injury site where they form the axonal growth cone with its lamellipodiae and filopodiae for axon elongation and path finding [9,22,23]. Bands of Büngner are formed by SC, which act as regenerative tubes, guiding the outsprouting axons by providing extracellular adhesion molecules and chemotactic cues [24]. The axons elongate in the direction of their target at a rate of about 1 mm per day, and are remyelinated with shorter internodes [1,9,16]. However, delayed nerve repair or very proximal injury may lead to chronic axotomy and SC denervation [1]. Chronic denervation begins around 3 months after injury and is characterized by a lack of neuronal contact and a failure of SC to maintain a pro-regenerative microenvironment [20,25]. Chronically denervated SC down-regulate the expression of neurotrophic factors and undergo apoptosis [22,26,27]. Depleted from trophic support, also the neuronal cell body and the distal nerve stump undergo molecular and morphological changes. Regeneration associated genes are down-regulated in the perikaryon, which undergoes apoptotic death, and the distal nerve stump is colonized by atrophic SC and fibroblasts, finally leading to failure of target re-innervation [27]. Also

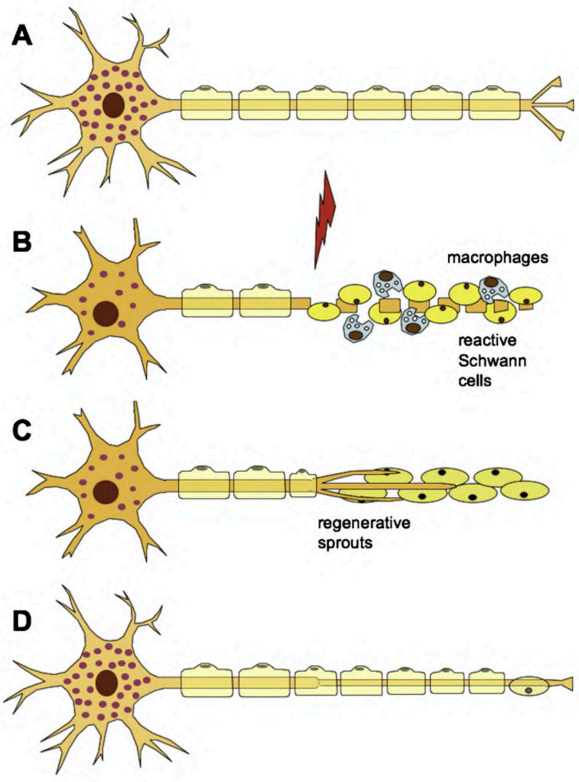

Figure 2. Degeneration and regeneration after peripheral nerve injury. **(A)** Normal neuron and nerve fiber. **(B)** Wallerian degeneration after axotomy. The distal axon and myelin sheaths are fragmentated, Schwann cells dedifferentiate into a regenerative phenotype and proliferate, macrophages invade the distal nerve segment and help to phagocytose debris. **(C)** Schwann cells line up in bands of Büngner in the distal nerve segment. The axonal growth cone extends lamellipodia and filopodia which advance depending on the different attractive and repulsive cues such as gradients of diffusible neurotrophic factors or permissive substrates of the extracellular matrix. **(D)** Successful axonal reconnection with the end organ and maturation and remyelination of the nerve fiber. *Figure from Allodi et al. [21]*

neuroma formation and false innervation leading to pain, hypersensibility and impairment of motor and sensory functions are common consequences of failed regeneration [28]. The target organ atrophies, followed by fibrosis of muscle fibers. The chance for functional recovery of denervated muscle tissue diminishes largely if re-innervation does not occur within one year [16]. Thus, correct and early treatment of nerve injuries is highly important in order to enable an acceptable recovery of motor and sensory functions [22].

3. NERVE REPAIR STRATEGIES – FROM PRAGMATIC TO FANCY

3.1. The Changing Paradigm in the Surgical Treatment of Non-Gap Nerve Injuries

In sharp and clean nerve dissections with minimal loss of tissue where a tension-free coaptation is possible, standard end-to-end neurorrhaphy with epineural suturing is performed [29,30]. Proper alignment of internal nerve fascicles and epineural vasculature results in better outcome [1,16]. However, epineural sutures create sites of inflammation and increased scarring, which are likely to hinder axonal regeneration [31]. Thus, the concept of suture-less nerve repair was proposed [31]. Several studies investigated the stabilizing effect of fibrin hydrogel on nerve coaptations [32,33], resulting in mixed outcomes. Equivalent reliability and even superior outcomes of suture-less coaptations due to decreased inflammation and scar tissue formation were observed by some authors compared to the standard epineural repair [34–36]. Others reported on insufficient stability provided by the fibrin gel, which led to gapping or even disconnection of the nerve ends [37,38]. A valid compromise might be to perform a minimum of epineural sutures with stabilization of the suture site by fibrin sealant. This technique resulting in increased tensile strength [39,40] has been applied successfully in vivo [34,41], despite concerns that fibrin might impede the axonal regeneration [42].

3.2. Surgical Repair Strategies for Nerve Gap Injuries

In case of a nerve gap where the nerve ends cannot be sufficiently mobilized for a tension-free coaptation, the treatment of choice is the interposition of an autologous nerve graft [43–45]. Donor nerves are dispensable sensory nerves such as the sural, or the medial and lateral antebrachial nerve [1]. The interposed nerve autograft undergoes Wallerian degeneration and thus presents as a guiding structure for proximally ingrowing axons. Moreover, it provides a growth stimulating environment with SC, neurotrophic factors (NTF)

and extracellular adhesion sites [1]. However, the harvest of a nerve autograft also comes along with several disadvantages. At the donor site a healthy nerve is sacrificed, which may lead to continued hyposensibility or neuroma formation in the grafted area. Also a second surgery site is created, which not only carries the risk of surgery-associated complications and suboptimal healing, but which also prolongs surgery time. At the recipient site, nerve size mismatch, and fibrosis from sutures and tissue handling may lead to unsatisfying regeneration [1,16].

The nerve transfer presents a surgical alternative to the nerve repair. The principle of this technique consists in bypassing the injured peripheral nerve, thus it allows a more flexible time window to perform surgery [45]. The nerve transfer is applied in very proximal nerve injuries for important motor or sensory nerve reconstruction. An expendable donor nerve is dissected, redirected and coaptated to the distal segment of a prioritized injured nerve [45]. In contrast to nerve grafting, in the nerve transfer usually only one neurorrhaphy site is needed, and it minimizes the regeneration distance for outsprouting axons in order to reach and re-innervate the end organ [1]. If transferred successfully, the recovered function may even be similar to the original muscle function. The outcome is enhanced if pure motor donors are joined to motor nerves and the same for sensory nerves [1]. Yet, the nerve transfer requires the availability of a suitable donor nerve.

3.3. Biological alternatives to the nerve graft

In order to overcome the disadvantages of the autologous nerve graft, several different autologous tissues have been investigated in the past for serving as a guiding structure. These include veins, arteries, skeletal muscles and tendons [7,11]. Despite reports about positive outcomes, the use of these alternatives never experienced a breakthrough in clinical practice. Mostly because of limited availability, complicated harvesting and their little potential in supporting nerve regeneration over a critical-sized nerve gap [11,46]. A promising alternative to autografts is currently seen in processed nerve allografts. With the help of decellularization processes any HLA-target tissue is removed, but leaving the internal structure and basal lamina of the nerve intact [47]. Thus, graft rejection and the need of any immunosuppression in the patient is avoided. The decellularized allograft furthermore provide the possibility to repopulate its matrix with regenerative cells [48,49]. Yet, the major challenge faced by allografts are ongoing improvements of these complex procedures [47].

3.4. Engineered nerve guidance conduits

Another intensively investigated treatment alternative to nerve autografts are nerve conduits (NC). NC are tubular structures used to bridge nerve gaps. After attaching the nerve ends intraluminally, the NC not only guides outsprouting axons, but also provides mechanical stability and prevents invasion of myofibroblasts and thus excessive fibrosis at the injury site [20,50]. In the past, attention was given to synthetic conduits out of non-degradable materials with silicone being clinically investigated [51,52]. However, due to its non-degradable nature compression symptoms, increased scarring and irritation at the wound site have been described. Furthermore, a second surgery was needed to remove the conduit [1,11,53]. Thus, current conduits are made of biodegradable polymers, which can be produced from a large variety of natural or synthetic sources [45], e.g. collagen, alginate, chitosan, silk fibroin and fibrin glue, lactic acid (PLA), glycolic acid (PGA), γ-hydroxybutyric acid (PHB), and ε-caprolactone (PCL). The significant advantage of using NC for nerve repair is to avoid the sacrifice of a healthy nerve and a second surgery site, easier surgery technique, reduced surgery time and thus cost effectiveness [54]. However, the challenge is to choose the proper material for a good conduit design. Several FDA- and CE- approved "off-the-shelf" conduits are already commercially available for clinical use [7,55]. Thus, the optimal NC has not yet been found. Such a conduit would need to incorporate several crucial characteristics such as 1) easy handling and adaptability, 2) biocompatibility, 3) flexible consistency with good mechanical stability, 4) selective permeability for regeneration- and vascularization-associated factors, and 5) timely resorption after having supported the sprouting axons [11,56,57].

Fibrin hydrogel is a promising biomaterial for nerve regeneration which is being investigated in various research groups. Fibrin is a protein naturally occurring in the human body during the coagulation cascade. It can be retrieved from individual patients for autologous use, or is commercially available [32,58]. In the surgical practice fibrin gel is widely accepted as hemostat, sealant and adhesive and has shown excellent biocompatibility [53,58–61]. The two main components of fibrin gel are a thrombin solution with calcium chlorid, and a fibrinogen solution with or without factor XIII and fibronectin, and an anti-fibrinolytic stabilization agent such as aprotinin [61–63]. The bio-mechanical properties of fibrin glue such as degradation, coagulation, malleability and permeability can be adapted by

modification of its components, and therefore allows a wide range of applications [62,64,65]. Fibrin sealant increased the tensile strength of reconstructed nerves when added around the coaptation site, allowing reduction of suture points [40,41]. Nerve conduits constructed out of fibrin gel were successful in supporting short- and long-term nerve regeneration in vivo [66–68]. In 10 mm short-gap nerve repair, outcomes in terms of axonal sprouting and muscle recovery were even equivalent to nerve autograft groups [67,68]. However, the use of fibrin nerve conduits (FNC) has not been translated into the clinical use for humans yet, despite its biocompatible behavior in the human body.

Following the example of nerve autografts, regeneration over a critical-sized nerve gap would also require a favorable microenvironment within the conduit, which would be able to support the neuroregenerative events for a sufficient time period until the nerve ends reconnect. Integration of luminal guidance structures, polymer coatings, immunosuppressive drugs, neurotrophic factors and stem cells are innovative strategies with the aim to improve the performance of nerve conduits [20,45,47]. The latter two are going to be discussed more closely in the following chapter.

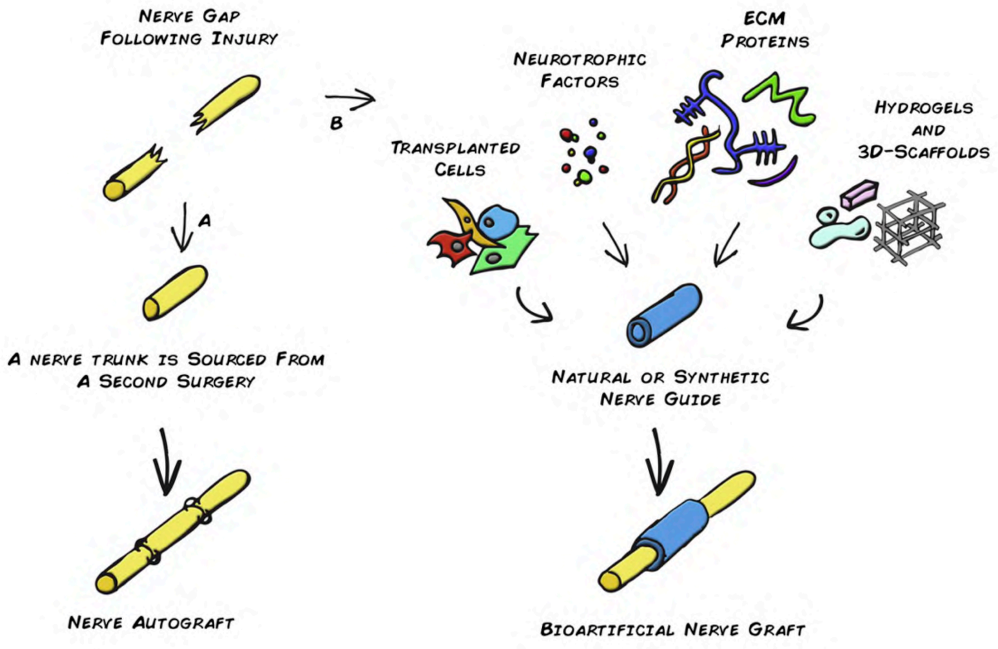

Figure 3. Repair strategies for the peripheral nerve gap-injury. **(A)** The "gold standard" autologous nerve graft. **(B)** The engineered bioartificial nerve conduit as an alternative nerve repair strategy. Such a conduit from natural or synthetic sources may be enriched with different factors for the axonal growth enhancement: 1) transplantable (stem) cells, 2) neurotrophic factors or pharmacological molecules, 3) extra-cellular matrix (ECM) proteins and 4) hydrogels or 3D scaffolds which may serve as guiding structures or cell/drug vehicles. *Figure from Faroni et al. [20]*

4. TRANSLATIONAL STRATEGIES FOR BIOACTIVE NERVE CONDUITS

4.1. Delivery of neurotrophic factors

NTF have been studied extensively for their important role during neuronal development and after nerve injury [69]. Physiologically, a basal expression of trophic signals is maintained by non-neuronal cells in order to ensure a homeostatic state for intact sensory, motor and autonomic neurons [22]. Following nerve injury, a range of NTF from neuronal and non-neuronal sources [23] is immediately upregulated for the support of neuronal survival, axonal regeneration and target organ re-innervation [7,22,69]. There are three main families of NTF: First, the neurotrophins, the probably most well-studied group, to whom belong NGF, BDNF, NT3 and NT4/5. Second, the GDNF family of neurotrophic factors ligands with GDNF, neurturin, artemin, and persephin. And third, the neuropoetic cytokines which generally refer to molecules of the interleukin-6 family and include IL-6, IL-11, LIF, oncostatin M, CNTF, cardiotrophin-1, neuropoietin and cardiotrophin-like cytokine [70,71]. They all act via binding to their specific high-affinity receptors, which can be found on neuronal cells and SC, as well as on ASC. The neurotrophins additionally share their common low affinity receptor p75. The exact composition of NTF in order to yield the best outcome however, still needs to be elucidated [72].

The other highly relevant questions address the adequate delivery dose, release kinetics and thus delivery method of soluble NTF from NC to the injury site. Controlled and sustained release of NTF seems to be desirable, even though the optimal dosing may depend on the specific growth factor [22,73,74]. Different delivery strategies exist to address this question: NTF incorporated into engineered micro- or nanovesicles, nanofiber scaffolds or polymer coating matrices, NTF-delivery via viral or plasmid vectors, mini-pumps or micro-injection ports [72,75–77], NTF-embedding via impregnation, and covalent or affinity based bonding to the conduit or conduit coating matrices [62,78–80]. A more natural way of providing NTF might be by secretion from transplanted neuroregenerative cells such as SC or mesenchymal stem cells (MSC).

4.2. Stem cell-based therapies

SC hold a crucial role in neuronal survival, axonal regeneration and remyelination after nerve injury [22,81,82]. The transplantation of SC to the injury site has proven beneficial to nerve

regeneration in various studies [24,48,75,83]. However, painful nerve biopsy and the sacrifice of a healthy nerve are required of the patient in order to harvest autologous SC. This is followed by rather challenging culture conditions in order to obtain sufficient amounts of transplantable cells, which results in significant treatment delay [84,85]. Therefore, the idea for stem cell-based therapies emerged, where therapeutic stem cells should be easily accessible and proliferate rapidly in culture.

It was discovered that MSC from bone marrow sources can be induced into SC-like cells (SCLC) under specific stimuli and enhance peripheral nerve regeneration [86–89]. To their disadvantage, their harvest requires painful biopsy as well; this is why attention turned to MSC obtained from adipose tissue [90,91]. Adipose tissue has been demonstrated to be a clinically viable source of multipotent cells, so called adipose-derived stem cells (ASC), which can be harvested in abundant quantity from liposuction or abdominoplasty procedures [91–93]. ASC also meet the requirements of fast and uncomplicated proliferation [92] and are able to differentiate towards mesenchymal and non-mesenchymal lineages similar to bone marrow-derived MSC (BMSC) [91,94,95]. In culture over several passages, ASC retain their mesenchymal stem cell properties and express a low immunogenic profile due to low expression of MHC class II antigens [96,97], which makes them suitable for allogeneic transplantation. Furthermore, they secrete an array of neurotrophic factors and cytokines [98–101] and can be differentiated towards a SC-like phenotype (SCLC), expressing markers such as S-100 and glial fibrillary acidic protein [98,99,101,102]. Direct comparison of SCLC derived from BMSC or ASC and SC in vivo showed similar potency for all three cell types in supporting nerve regeneration [85]. However, a significant disadvantage limiting the clinical use of SCLC is the lengthy differentiation process, which requires up to three weeks. Furthermore, it has been shown that SCLC derived from ASC rapidly return to stem cell-like characteristics after withdrawal of the differentiation medium [103]. Thus, applicability and relevance of SCLC for the clinical routine might be questioned, especially since several studies suggest a similar therapeutic potential for ASC and SCLC derived from ASC [104–106]. There are two possible mechanisms explaining the regenerative potency of ASC in response to local stimuli at the nerve injury site: The first hypothesis suggests in vivo trans-differentiation of ASC into SCLC [102,105,107,108]. The other hypothesis sees the neurotrophic potential of ASC in their secretome, which contains a wide range of molecular factors [98,100,109]. Furthermore, transplanted ASC may enhance recruitment of

endogenous SC to the injury site [104]. Thus, there is a clear need for exploring the regenerative capacity of ASC involving only minimal manipulation procedures in vitro [8].

Another line of research represents the delivery of cells within a NC. For the transplantation of cells to the injury sites, NC can be used as scaffolds for cell seeding [24]. Biological extracellular matrices such as fibrin, collagen, laminin and fibronectin provide structural support for stem cell adhesion [24,110,111]. Stem cells loaded in fibrin scaffolds were able to adhere, spread and proliferate [112–114]. However, the most optimal delivery system remains to be determined. Particularly for FNC only few methods have been evaluated and no systematic comparison was reported so far. One method is to inject a fibrin solution loaded with cells intraluminally [115]. An alternative approach is to integrate the cell-loaded fibrin solution into the main structure of the FNC's wall by polymerization [116]. The last method is to suspend the cells in a carrier medium and to place them into the lumen of the conduit [117].

5. IMPORTANCE OF THE RESEARCH ON PERIPHERAL NERVE REPAIR AND REGENERATION

Gold standard treatments for the peripheral nerve repair have experienced immense improvements through advanced microsurgical techniques. However, these procedures are associated with inevitable co-morbidities. Thus, multiple lines of research have emerged with the aim to tackle these challenges with a variety of approaches targeting different levels and structures of the injury. However, the optimal alternative treatment strategy which matches the regenerative level of the standard techniques has not yet been found.

6. RESEARCH OBJECTIVES

This MD-PhD work aimed at investigating the potential of novel strategies for peripheral nerve repair and regeneration. Ex-vivo stimulation of ASC by NTF might present an innovative approach for stem cell-based therapies. Furthermore, fibrin hydrogel is a promising biomaterial which provides a suitable scaffold for stem cell delivery. It has also been used successfully in shape of NC for bridging nerve gap-injuries in vivo. However, neither has the most beneficial delivery route been established yet, nor has the suitability of FNC been evaluated for clinical use. The present thesis explores these questions in different experimental settings by using translational approaches. The specific objectives for each manuscript are stated in detail in the following paragraphs.

Manuscript 1: Bioactive fibrin nerve conduits

Objective 1: Evaluation of the potency of ASC in response to specific stimuli of NGF or VEGF for promoting the axonal regeneration *in vitro* and *in vivo*.

Objective 2: Investigation of the influence of the stem cell delivery route, i.e., FNC assisted intramural vs. intraluminal ASC loading on early nerve regeneration *in vivo.*

Manuscript 2: Human adipose stem cells with enhanced neurotrophic capacity

Objective 1: Providing a comparative *in vitro* assay analyzing the neurotrophic potency of ASC in response to stimulation by NGF, BDNF, NT3, NT4, GDNF or CNTF.

Objective 2: Analysis of the neuroregenerative events taking place in ASC and sensory neuronal cells upon stimulation *in vitro*.

Manuscript 3: Fibrin hydrogel for the *clinical* peripheral nerve repair

Objective 1: In non-gap digital nerve injuries, proving non-inferiority in clinical outcomes and patient satisfaction for the experimental (epineural suture with fibrin sealant) repair compared to the standard (epineural suture) repair with a one-year follow-up after surgery.

Objective 2: In critical-sized nerve gap digital injuries, proving non-inferiority in clinical outcomes and patient satisfaction for the experimental (FNC) repair compared to the standard (autologous nerve graft) repair with a one-year follow-up after surgery.

7. CONTRIBUTIONS BY THE MD-PHD STUDENT

The projects of manuscript 1 and 2 were originally designed by PD Dr. Srinivas Madduri and me. With the help and advices of my co-authors and under the excellent supervision of PD Dr. Srinivas Madduri and Prof. Daniel Kalbermatten I managed these projects, performed all the experiments and prepared the first manuscripts. I was and still am involved in the revision and journal submission and publication processes. The clinical study of manuscript 3 was initiated before I started my MD-PhD studies. For the first 2 years of my studies I was involved in the management of the patient follow-ups and the data acquisition. During the last year of my studies I was, together with Prof. Daniel Kalbermatten, the main responsible person for the management and conduct of this study. I prepared the present manuscript and will continue to work on this clinical trial until its completion and publication.

Detailed information about the involvement of the co-authors in the respective projects is given after each manuscript.

MANUSCRIPT 1

Ex-Vivo Stimulation of Adipose Stem Cells by Growth Factors and Fibrin-Hydrogel Assisted Delivery Strategies for Treating Nerve Gap-Injuries

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ABSTRACT

Peripheral nerve injuries often result in lifelong disabilities despite advanced surgical interventions, indicating the urgent clinical need for effective therapies. In order to improve the potency of adipose-derived stem cells (ASC) for nerve regeneration, the present study focused primarily on ex-vivo stimulation of ASC by using growth factors, i.e., nerve growth factor (NGF) or vascular endothelial growth factor (VEGF) and secondly on fibrin-hydrogel nerve conduits (FNC) assisted ASC delivery strategies, i.e., intramural vs. intraluminal loading. ASC were stimulated by NGF or VEGF for 3 days and the resulting conditioned medium (CM) was subsequently evaluated in an in vitro axonal outgrowth assay. For the animal study, a 10 mm sciatic nerve gap-injury was created in rats and reconstructed using FNC loaded with ASC. CM derived from NGF-stimulated ASC promoted significant axonal outgrowth from the DRG-explants in comparison to all other conditions. Thus, NGF-stimulated ASC were further investigated in animals and found to enhance early nerve regeneration as evidenced by the increased number of β-Tubulin III+ axons. Notably, FNC assisted intramural delivery enabled the improvement of ASC's therapeutic efficacy in comparison to the intraluminal delivery system. Thus, ex-vivo stimulation of ASC by NGF and FNC assisted intramural delivery may offer new options for developing effective therapies.

Keywords: adipose stem cells; neurotrophic factors; growth factors; peripheral nerve injuries; fibrin nerve conduits; hydrogels; stem cells delivery; axonal regeneration; Schwann cells

1. INTRODUCTION

Peripheral nerve injuries often result in loss of sensory and motor functions due to lack of effective therapeutic strategies, thus there is a great clinical need for developing new therapies [118,119]. Schwann cells (SC) play a crucial role in neuronal survival, axonal regeneration and re-myelination [22,81] by secreting an array of molecular signals, neurotrophic factors (NTF) and extracellular matrix proteins [82]. Furthermore, SC generate bands of Büngners for topographical guidance and path finding of the regenerating axons [92]. However, the therapeutic use of SC is hampered due to the problems associated with harvesting the cells from healthy nerves and resulting co-morbidities [85]. Therefore, the need for stem cell-based therapies emerged for treating nerve injuries.

Therapeutic stem cells should be easily accessible and undergo rapid proliferation and differentiation in vitro under controlled conditions. Mesenchymal stem cells (MSC) can differentiate into SC-like cells (SCLC) under specific stimuli and enhance peripheral nerve regeneration [86–89]. Not limiting to the source of bone marrow, MSC with multi-lineage capacity can also be obtained from adipose tissue, dental pulp, umbilical cord blood and Wharton's jelly of umbilical cord [90,91,120,121]. Adipose-derived stem cells (ASC) are easily accessible in large quantities from fat-tissue enabled by liposuction or abdominoplasty [91–93]. ASC proliferate rapidly [92] and possess a multi-lineage capacity, i.e., adipocytes, osteoblasts, chondrocytes [91,94,122]. Furthermore, ASC retain their mesenchymal potency over long-term culture [92,122] and promote the nerve regeneration similar to bone marrow stem cells [85]. In allogeneic transplantation, usage of ASC, like any other adult stem cells, benefits from their hypo-immunogenicity or immune-privilege by virtue of the reduced expression of HLA-DR class II histocompatibility antigens [96,97]. Thus, ASC may enable the development of "off-the-shelf therapies" for promoting nerve regeneration. Within this context, the neurotrophic potency of ASC and their delivery approaches play a crucial role in improving the therapeutic efficacy for peripheral nerve injuries.

As shown by many studies, factors that are present in the secretome of ASC promote axonal regeneration both in vitro and in vivo. When co-cultured with motor neuron-like cells or dorsal root ganglion (DRG) sensory neurons, ASC enhanced the neurite number and outgrowth length [102,123,124]. In line with these findings, several animal studies reported enhanced axonal regeneration and sensory-motor nerve conduction, when ASC were transplanted in polymeric nerve conduits (NC) or fibrin-hydrogel nerve conduits (FNC)

[116,125,126]. Improved axonal growth and elongation was found in FNC loaded with ASC in comparison to empty FNC [115,125,127]. However, the optimal delivery route for ASC still remains to be established in the context of peripheral nerve reconstruction. Various studies involving ASC therapy employed different cell delivery routes, i.e., injection into the lumen, dispersion within the fibrin conduit wall, dispersion within the lumen of fibrin matrix and coating on the luminal surface [85,104,115–117,123]. However, the therapeutic efficacy of the cells was scarcely correlated with delivery routes and the carrier matrix in the context of nerve regeneration. Thus, the ineffective and incomplete outcome achieved so far by using the ASC therapy can be largely attributed to various structural and biochemical aspects of the microenvironment at the injury site that were inadequately orchestrated through different delivery routes. Moreover, studies focusing on the understanding of the spatiotemporal influence of the cell carrier matrix on the efficacy of therapeutic cells are largely missing. Therefore, there is a clear need to investigate systematically the impact of important local cell delivery strategies on nerve regeneration and to establish effective options.

The most important soluble factors known to support the neuronal survival and axonal regeneration following traumatic nerve injury are NTF [7,20,22,76]. Nerve growth factor (NGF), amongst other NTF, specifically promotes survival and regeneration of sensory neurons by binding to the high-affinity trk-A receptors [74]. Vascular endothelial growth factor (VEGF) on the other hand is a potent angiogenic factor, which promotes the proliferation of endothelial cells, and the formation and permeability of vascular structures [128]. Nevertheless, several studies demonstrated VEGF for having neurotrophic activities that were mediated either by flk-1 and flt-1 receptor binding or by enhanced vascularization [128–131].

Same as for ASC, various studies reported on improved nerve regeneration supported by exogenously administered NTF [74,76,78,132]. Despite these findings, the growth-promoting effects of ASC in response to a specific growth factors' stimulus largely remain elusive in the context of nerve regeneration. However, rapid upregulation of NGF and VEGF after traumatic nerve injury [7,22] clearly indicates the clinical significance for exploring the therapeutic impact of ASC following growth factors' stimulation in the context of axonal regeneration.

We hypothesize an improvement in the neurotrophic capacity of ASC in response to exogenous growth factors stimulation. Furthermore, a novel design of the fibrin hydrogel nerve conduits would facilitate discrete options for cell delivery and for the enhancement of their therapeutic efficacy. Thus, the present study evaluated the capacity of ASC in response to specific stimuli of NGF or VEGF for promoting the axonal regeneration in vitro and in vivo. The influence of the stem cell delivery route, i.e., FNC assisted intramural vs. intraluminal ASC loading on early nerve regeneration was investigated in rats by using a 10 mm sciatic nerve gap-injury model.

2. MATERIALS AND METHODS

2.1. Isolation and Culture of Adipose Stem Cells (ASC)

All in vitro studies were conducted in accordance with the local veterinary commission in Basel, Switzerland (No. 2925). Visceral adipose tissue was harvested from adult Sprague-Dawley rats and processed under sterile conditions as described earlier [102]. Briefly, the fat tissue was rinsed in 0.01 M phosphate-buffered solution (PBS), minced, and resulting tissue was digested with 0.15% (w/v) Type I Collagenase (Gibco Life Technologies, Cat. No. 17100017) for 1 h at 37 °C and centrifuged for 5 min at 1500 rpm and 4 °C. The pellet was re-suspended in growth medium (GM) i.e., Dulbecco`s Modified Eagle`s Medium (DMEM, Gibco, Cat. No. 41965039) supplemented with 10% Foetal Bovine Serum (PAN-Biotech, EU-approved, Cat. No. P40-47500) and 1% Penicillin/Streptomycin (BioConcept, Cat. No. 4-01F00-H). Isolated ASC were seeded at a density of 3000 cells/cm^2 and expanded at 37 °C with 5% CO2 in a humid atmosphere; the GM was changed every 72 h. Cells were passaged using 0.25% Trypsin-EDTA (BioConcept, Cat. No. 5-51F00-H) at 90% confluence and resulting cells at passage 2 (P2) or 3 (P3) were used for the experiments.

2.2. ASC Characterization

Rat ASC (P2) were seeded on 24-well plates for characterization. Cells were fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 10 min and permeabilized and blocked in 1% normal goat serum (NGS) in PBS (i.e., dilution buffer) for 60 min at RT. ASCs were incubated overnight at 4 °C with the human mesenchymal stromal cell markers monoclonal mouse anti-CD44 (1:1000), monoclonal mouse anti-CD90 (1:200), monoclonal mouse anti-CD105 (1:200) and monoclonal rabbit anti-CD29 (1:100), and the hematopoietic

marker polyclonal rabbit anti-CD45 (1:500) (Abcam, Cat. No. ab93758). Cells were then washed in PBS and incubated for 60 min at RT with the secondary antibody goat anti-mouse Alexa Fluor 488 (1:500, Abcam, Cat. No. ab150109) and goat anti-rabbit Alexa Fluor 488 (1:500, Abcam, Cat. No. ab150061), and Hoechst 33258 nuclear staining (1:1000, Sigma Aldrich, Cat. No. 94403). Subsequently, digital images were acquired at 20× magnification (numerical aperture 0.45) by using a Nikon Eclipse Ti2 fluorescent inverted microscope (Nikon Eclipse Ti2-E, -E/B, Nikon Corporation, Japan) and a Photometrics prime 95B 25 mm camera (Teledyne photometrics, Tucson, AZ, USA). The images were automatically stitched by the Nikon NIS-Elements AR image analysis software (NIS- Elements AR Analysis 5.11.00 64-bit, Laboratory Imaging, spol. s.r.o., Praha, Czech Republic). Furthermore, immunostaining images of ASC for various markers were analyzed quantitatively using ImageJ.

2.3. Isolation of Chicken Embryonic Dorsal Root Ganglions (DRG)

Fertilized chicken eggs were obtained from Gepro Geflügelzucht AG (Flawil, Switzerland). The eggs were shipped at ambient temperature and incubated at 37.8 ± 0.2 °C under 100% relative humidity for 10 days (E10). After incubation, the eggs were cleaned with 70% ethanol and opened under a laminar airflow cabinet to collect the embryos. E10 embryos were dissected following a standard dissection protocol under a stereomicroscope [74]. DRG-explants were collected from the lumbar part of the spine and transferred to GM for cell culture.

2.4. ASC Stimulation and Conditioned Medium Harvest

ASC were seeded on 24-well plates at a density of 13,000/cm^2 and 500 µL of GM was added with or without supplementation of exogenous growth factors, i.e., 10 ng/mL of recombinant human NGF or recombinant mice VEGF, as indicated in the experimental design. NGF (Cat. No. 256-GF) and VEGF (Cat. No. 493-MV) were obtained from R&D Systems (Minneapolis, MN, USA). ASC were cultured for 72 h and no growth medium was exchanged during culture to enrich the secretome. After 72 h of enrichment, the resulting conditioned medium (CM) enriched with secretome was collected and used for subsequent experiments.

2.5. Experimental Design In Vitro

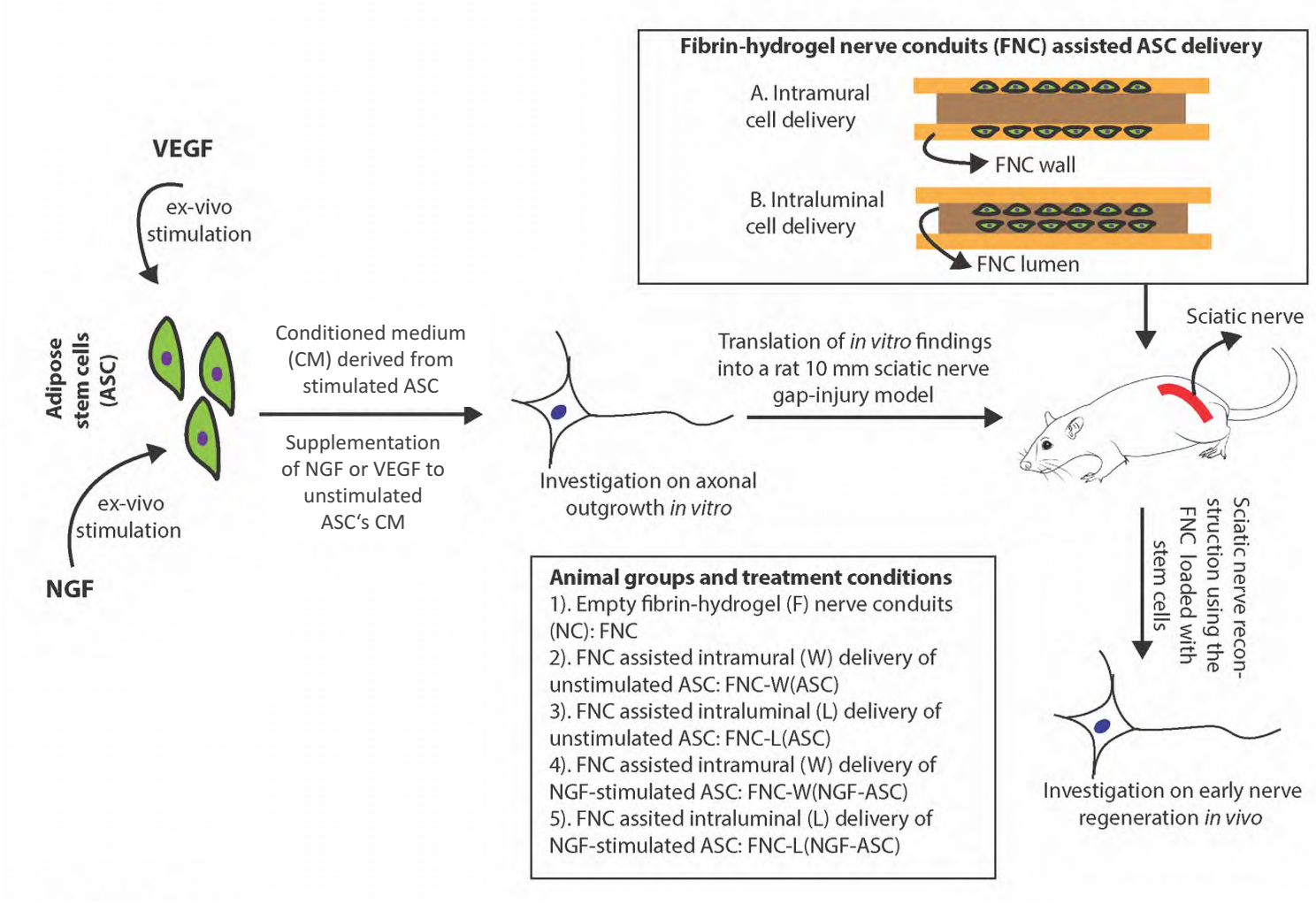

Figure 1. Ex-vivo stimulation of adipose stem cells using growth factors, and fibrin-hydrogel nerve conduits' assisted stem cell delivery strategies for enhancing the axonal regeneration.

As illustrated in Figure 1, ASC were stimulated by NGF (NGF-ASC) or VEGF (VEGF-ASC) or without growth factor (ASC) for 72 h and resulting conditioned medium (CM), i.e., CM-NGF-ASC, CM-VEGF-ASC and CM-ASC was subsequently used for DRG assay for 48 h. When the ASC were not stimulated prior to DRG assay, the CM derived from the ASC was either supplemented with NGF (CM-ASC+NGF) or VEGF (CM-ASC+VEGF) or no growth factors (CM-ASC) at the time of DRG seeding. As a control, culture conditions with NGF alone (NGF) or VEGF alone (VEGF) or without growth factors (no GF) were used. In all cases, growth factors were applied at 10 ng/mL based on preliminary experiments [74]. For the axonal outgrowth assay, DRG-explants were seeded at a density of one per well onto 24-well plates. Cultures were maintained in a humid atmosphere at 37 °C and 5% CO^2 for 48 h and images were

captured at 5x and 10x magnification. In total, 3 independent experiments resulting in a total of 8 DRG-explant cultures for each experimental condition were performed.

2.6. Immunocytochemistry of DRG Cultures

After 48 h, DRG cultures were observed under a microscope and bright-field images with phase-contrast were taken at 5x magnification using a Zeiss Axio Vert.A1 inverted fluorescent microscope (Carl Zeiss AG, Jena, Germany) and a Zeiss AxioCam MRc camera (Carl Zeiss AG, Jena, Germany). DRG-explants were then fixed in 4% PFA at RT for 10 min, and then permeabilized and blocked in PBS containing 0.1% Triton X-100 and 1% BSA (i.e., dilution buffer) for 60 min at RT. For immunocytochemistry, the cultures were incubated overnight with the following primary antibody at 4 °C: monoclonal mouse anti-β-Tubulin III (1:1000, Sigma-Aldrich, Cat. No. T8578) for axons. The cultures were then washed in PBS and incubated with the following secondary antibody: sheep anti-mouse Cy3 (1:500, Sigma Aldrich, Cat. No. C2181) and Hoechst 33258 nuclear staining (1:1000, Sigma Aldrich, Cat. No. 94403) for 60 min at RT. Subsequently, digital images were acquired at 10x magnification (numerical aperture 0.45) by using a Nikon Eclipse Ti inverted fluorescent microscope (Nikon Eclipse Ti-E, -E/B, Nikon Corporation, Japan) and a Photometrics prime 95B 25 mm camera.

2.7. Quantitative Measurements of Axonal Outgrowth

DRG-explant cultures were analyzed for axonal length and axonal area in an automated manner with a standardized analysis mask created using the Nikon NIS-Elements AR image analysis software. The DRG area was defined in the Hoechst channel and the intensity threshold was set to be 45. The area occupied by axonal outgrowth was evaluated in the Cy3 channel and the intensity was set to be 65. For accurate axonal area measurements, no binary processing for “fill holes” was selected. For analysis of the axonal length, on the other hand, binary processing for “fill holes” was used. A binary operation expression was used for defining the origin of axonal outgrowth from DRG as well as axonal endpoints. The shortest perpendicular distances from the axonal growth origin to the endpoints were measured and the output was displayed automatically.

2.8. Stimulated ASC for Animal Studies

ASC were harvested and cultured as explained earlier. Briefly, ASC were cultured in 75 cm^2 flasks at a density of 13000 cells/cm^2 and stimulated by NGF 10 ng/mL for 72 h prior to transplantation.

2.9. Fibrin-Hydrogel Nerve Conduits (FNC)

FNC were prepared according to the manufacturer's instructions as described earlier (Tisseel Kit VH 1.0, Baxter, SA, USA) [66,67]. Briefly, Tisseel provides a fibrinogen solution containing fibrinogen 100 mg/mL, factor XIII 0.6–10 IU/mL, plasminogen 40–120 mg/mL, aprotinin synthetic 3000 KIU/mL, and a thrombin solution 500 IU/mL with calcium chloride 40 µmol/mL. Fibrin conduits measuring 14 mm in length, 1 mm in wall thickness and 2 mm in lumen were produced for bridging a 10 mm nerve gap-injury. The thrombin 500 IU/mL was diluted in sterile water to 30 IU/mL. Equal amounts of the diluted thrombin and the fibrinogen solution were mixed directly into a silicone mold pre-set with a stainless steel lumen and incubated for 30 min at 37 °C for polymerization. Resulting FNC constructs were stored in sterile PBS at 4 °C for a maximum of 24 h before animal experiments.

2.10. Intramural Delivery of ASC

About 2 million NGF-stimulated ASC or unstimulated ASC were incorporated into the wall of the conduit, the resulting fibrin conduits were designated as FNC-W(NGF-ASC) and FNC-W(ASC) respectively. Immediately before transplantation, cells were trypsinized and suspended in a volume of 540 µL Fibrinogen. Further, 60 µL of thrombin solution 30 IU/mL was added to the ASC-Fibrinogen suspension and the resulting solution was cast into the FNC.

2.11. Intraluminal Delivery of ASC

About 2 million NGF-stimulated ASC or unstimulated ASC were incorporated into the lumen of the conduit, the resulting fibrin conduits were designated as FNC-L(NGF-ASC) and FNC-L(ASC) respectively. Cells were suspended in the final volume of 20 µL of Fibrinogen solution (25 mg/mL). Further, 20 µL of thrombin was added to the ASC-Fibrinogen suspension. The

resulting solution was injected into the lumen of the FNC and incubated for 30 min at 37 °C for polymerization.

2.12. Surgical Procedure and Experimental Groups In Vivo

All the studies were conducted in accordance with the local veterinary commission in Basel, Switzerland (No. 2925). About 12-week old female Sprague-Dawley rats weighing 250–300 g (Janvier, Mayenne, France) were used for the experiment. A total of 36 rats were operated and randomly categorized into 6 groups: 1) autograft, 2) fibrin-hydrogel nerve conduit without cells, i.e., FNC, 3) FNC assisted intramural delivery of unstimulated ASC, i.e., FNC-W(ASC), 4) FNC assisted intramural delivery of NGF-stimulated ASC, i.e., FNC-W(NGF-ASC), 5) FNC assisted intraluminal delivery of unstimulated ASC, i.e., FNC-L(ASC), 6) FNC assisted intraluminal delivery of NGF- stimulated ASC, i.e., FNC-L(NGF-ASC). All surgical procedures were performed under general anesthesia with 3% isoflurane. Routinely, Buprenorphine (Temgesic ®, 0.3 mg/mL, 0.05 mg/kg, Indivior AG, Baar, Switzerland) was administered before and after the surgery. The left sciatic nerve was approached dorsally using a gentle spreading technique of the gluteus muscle. The sciatic nerve was transected 5 mm distal to the gluteal branch and nerve ends were inserted 2 mm into the fibrin conduit and fixed to the conduit by a single epineural suture (9/0 nylon, S&T). Muscles and fascia layers were closed with a single resorbable stitch (5/0 Vicryl, Ethicon) and the skin was closed by a continuous running suture (5/0 Vicryl, Ethicon). For analgesia, the animals received subcutaneous injections of Meloxicam (Metacam ®, 2 mg/mL, Boehringer Ingelheim GmbH, Basel, Switzerland) 2 mg/kg BW postoperatively, and then 1 mg/kg BW every 24 h for two days. Further, Paracetamol (Becetamol ®, 100 mg/mL, Gebro Pharma AG, Liestal, Switzerland) 50mg/kg BW/day was added to the drinking water for 7 days.

2.13. Tissue Processing and Immunohistochemistry

At 4 weeks post-operation, all the animals were euthanized by CO^2 and regenerated nerve tissue was harvested with the proximal and distal nerve stumps. The harvested nerves were embedded in OCT freezing media (Tissue-Tek, Sakura, Japan) and flash-frozen in liquid nitrogen through 2-methylbutane (Sigma Aldrich, Cat. No. M32631). Nerve cross-sections were prepared by cryostat from the middle, distal and far distal part of the explanted nerve tissue as shown in Figure 2. The middle sections were taken right in the middle of the

explanted conduit, the distal sections at the distal suture point, and the far distal sections 5mm distal to the distal suture. Serial 5 µm thick tissue sections were prepared onto slides (Superfrost plus, Menzel-Gläser, Braunschweig, Germany) and stored at −80 °C. For staining, every second section was processed. First, the slides were fixed in 4% PFA for 10 min and washed in distilled water and then blocked using dilution buffer for 60 min at RT. Slides were then incubated overnight at 4 °C with the following primary antibodies: monoclonal mouse anti-β-Tubulin III (1:1000, Sigma Aldrich, Cat. No. T8578) and polyclonal rabbit anti-S100 (1:100, abcam, Cat. No. ab76729). After rinsing in PBS, secondary sheep anti-mouse antibodies Cy3 (1:500, Sigma Aldrich, Cat. No. C2181), donkey anti-rabbit Alexa Fluor 488 (1:500 abcam, Cat. No. ab150061) and Hoechst 33258 (1:1000, Sigma Aldrich, Cat. No. 94403) were applied for 60 min at RT. The slides were mounted using ProLong Gold (Invitrogen, Cat. No. P36930) and digital images were acquired using a Nikon Eclipse Ti2 inverted fluorescent microscope and a Photometrics prime 95B 25 mm camera at 10x (numerical aperture 0.45), 20x (numerical aperture 0.75) and 40x (numerical aperture 0.95) magnification.

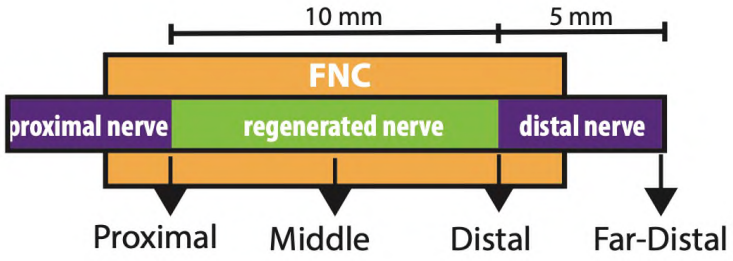

Figure 2. Anatomical segmentation representing the histological analysis of the reconstructed nerves.

2.14. Histological Analysis

Following immunofluorescence staining, digital images of 20x magnification were acquired and used for quantitative analysis of anatomical structures. For measuring the axonal density and area occupied by SC, an automated program was performed using the standardized analysis mask created by Nikon NIS-Elements AR image analysis software. Axonal count and nerve area values were used for calculation of axonal density. Similarly, the area occupied by SC was given in reference to the nerve area.

2.15. Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) following Bonferroni procedure with post hoc multiple comparisons using SPSS (version 15.0; SPSS, Chicago, IL, USA). Values with $p < 0.05$ were considered significant.

3. RESULTS

3.1. Characterization of Isolated ASC

ASC were isolated, cultured, and resulting cells were characterized phenotypically by immunocytochemistry. ASC were found to be positive for mesenchymal marker CD29 (87%), CD44 (78%), CD90 (81%) and CD105 (85%), and negative for hematopoietic marker CD45 (Figure S1).

3.2. Stem Cell Derived Conditioned Medium and Axonal Growth In Vitro

Consistent with our previous reports [74], DRG explants exhibited an important and dense axonal outgrowth in response to NGF-stimulation (Figure 3A). Quantitative measurements of axonal outgrowth, i.e., axonal length (in µm) and axonal area (in mm^2) resulted in 307 ± 110 and 1.05 ± 0.37 (Figure 3B and 3C). In contrast to NGF, stimulation with VEGF or without growth factors (no GF) resulted in only minimal axonal length, i.e., 85 ± 55 and 66 ± 45 (Figure 3B), which are consistent with axonal area measurements, i.e., 0.14 ± 0.10 and 0.10 ± 0.05 (Figure 3C) respectively.

Interestingly, CM-NGF-ASC enhanced the significant axonal outgrowth, i.e., 657 ± 224 and 1.76 ± 0.65 (Figure 3D and 3E). In the case of CM-ASC, no significant axonal outgrowth could be observed, i.e., 80 ± 56 and 0.083 ± 0.039 (Figure 3F). Together these observations clearly indicate the significantly enhanced potency of ASC in response to the NGF-stimulation for promoting axonal regeneration in vitro (Figure 3D, 3E and 3F).

In contrast to NGF conditions, CM-VEGF-ASC did not result in the enhancement of axonal outgrowth, i.e., 161 ± 55 and 0.111 ± 0.032 (Figure 3E). These observations indicate no significant improvement of ASC's potency in response to VEGF-stimulation for supporting axonal regeneration in vitro (Figure 3D, 3E and 3F).

In line with CM-NGF-ASC, CM-ASC+NGF culture condition resulted in a robust axonal outgrowth, i.e., 569 ± 86 and 1.98 ± 0.53 (Figure 3G, 3H and 3I). Together these results underline the important role of NGF for promoting axonal regeneration (Figure 3B, 3E and 3H). In contrast to these results, the CM-ASC+VEGF culture condition did not elicit synergy as evidenced by axonal outgrowth measurements, i.e., 181 ± 51 and 0.22 ± 0.077 (Figure 3H and Figure 3I).

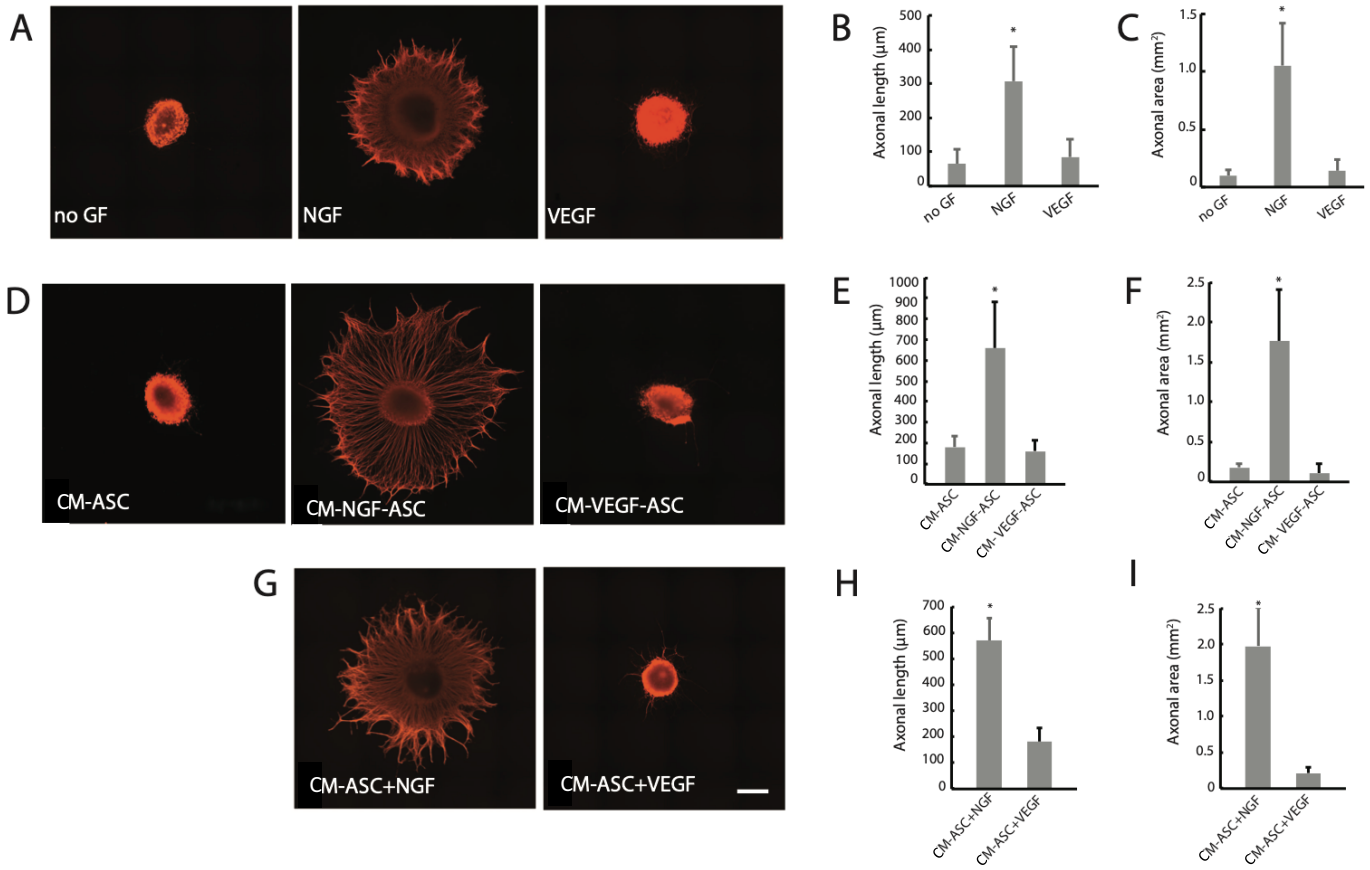

Figure 3. Axonal outgrowth from DRG-explants in vitro. **(A)** Microphotographs of DRG-explant cultures treated with growth factors. **(B)** Quantitative measurements of axonal length (µm). **(C)** Quantitative measurements of the axonal area (mm^2). **(D)** Microphotographs of DRG-explant cultures that were treated with conditioned medium (CM) derived from unstimulated ASC (CM-ASC) or NGF-stimulated ASC (CM-NGF-ASC) or VEGF-stimulated ASC (CM-VEGF-ASC). **(E)** Quantitative measurements of axonal length (µm). **(F)** Quantitative measurements of the axonal area (mm^2). **(G)** Microphotographs of DRG-explant cultures that were treated with ASC's CM in combination with exogenous NGF (CM-ASC+NGF) or VEGF (CM-ASC+VEGF). **(H)** Quantitative measurements of axonal length (µm). **(I)** Quantitative measurements of the axonal area (mm^2). The red staining is β-Tubulin III+ for regenerating axons. For images **(A)–(I)**, the scale bar represents 500 µm and the bars represent the mean ± SD of n = 8. Significant differences at * $p < 0.05$ are indicated in comparison to all other treatment groups.

Group	Axonal Length (μm)	Axonal Area (mm^2)
no GF	0.066 ± 0.045	0.099 ± 0.049
NGF	0.307 ± 0.110	1.051 ± 0.371
VEGF	0.085 ± 0.055	0.141 ± 0.095
CM-ASC	0.080 ± 0.056	0.083 ± 0.039
CM-NGF-ASC	0.657 ± 0.224	1.760 ± 0.649
CM-VEGF-ASC	0.161 ± 0.055	0.111 ± 0.032
CM-ASC+NGF	0.569 ± 0.086	1.976 ± 0.527
CM-ASC+VEGF	0.181 ± 0.051	0.215 ± 0.077

Table 1. Quantitative measurements of the axonal length (μm) and of the axonal area (mm^2) expressed as mean ± SD.

3.3. Stimulated Stem Cells and Delivery Route Impacted Early Nerve Regeneration

Considering the in vitro findings on the enhanced potency of ASC in response to NGF-stimuli, NGF-ASC were further investigated in rats for treating a 10 mm sciatic nerve gap-injury. The impact of the different ways of delivering ASC on early nerve regeneration, i.e., intramural vs. intraluminal delivery, was also analyzed (Figure 1).

As depicted in Figure 4, histological recovery of the treated animals was measured by analyzing the β-Tubulin III+ axons (Figure 4 and 5) and S100 + SC structures (Supplementary Figure S2A, B) from the middle, distal and far-distal parts of the regenerated nerve tissue. In the middle part of the fibrin conduit, axonal regeneration (i.e., number of axons/mm^2) resulted in the order of 6190 ± 2061, 5260 ± 1257, 3075 ± 1432, 2614 ± 743, 1810 ± 629 and 2197 ± 1478 for autograft, FNC-W(NGF-ASC), FNC- W(ASC), FNC-L(NGF-ASC), FNC-L(ASC) and FNC treatment groups respectively. These results reveal the enhanced axonal regeneration supported by the NGF-stimulated ASC. On the other hand, the superior performance of the intramural stem cell delivery was evident compared to the intraluminal delivery route (Figure 4 and 5). Together, these results reveal the autograft matching performance of NGF-stimulated ASC in combination with the intramural delivery route i.e., FNC-W(NGF-ASC). Furthermore, the general tendency of enhanced axonal regeneration supported by the FNC-W(NGF- ASC) is evident in the distal and far-distal segments in comparison to all other

treatment groups (Figure 4 and 5), although the differences between some of the groups were not statistically significant (Figure 5).

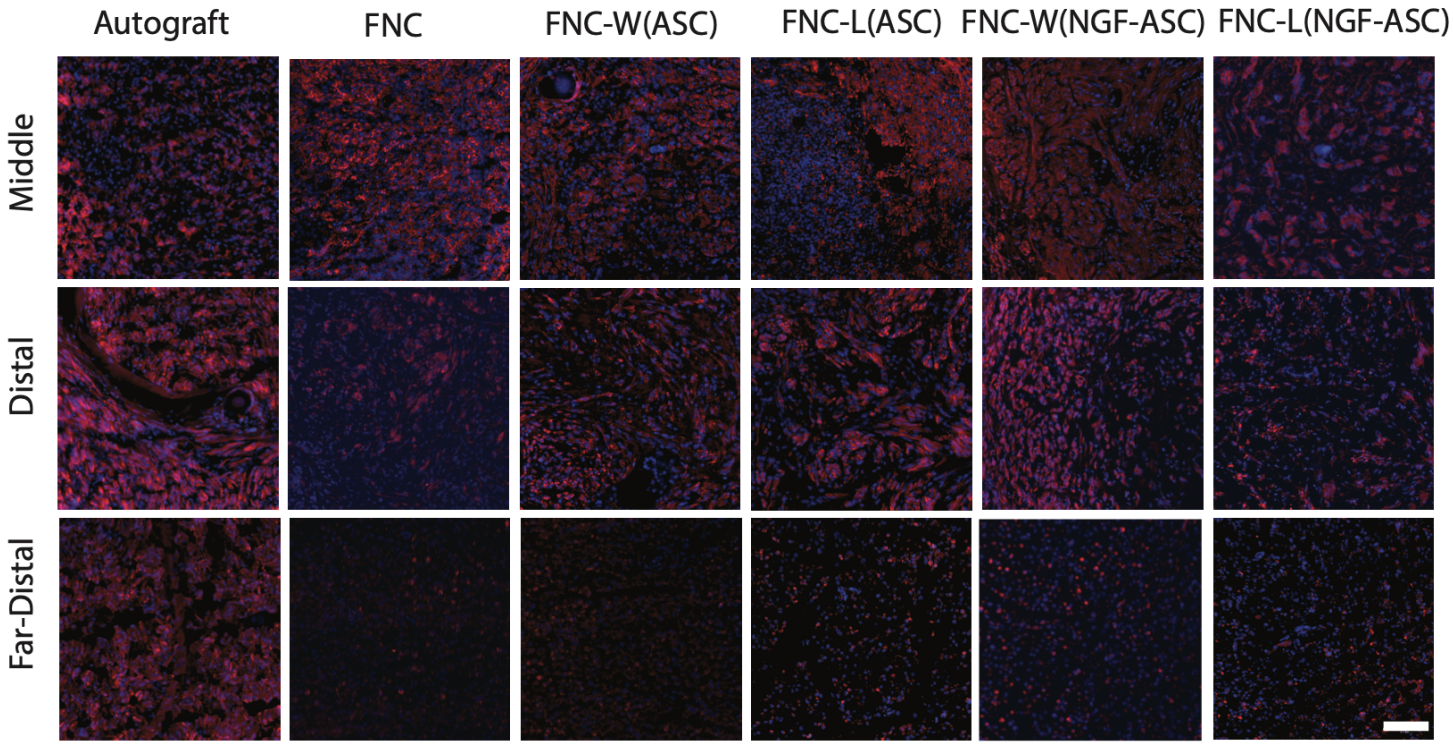

Figure 4. Fibrin assisted delivery of (stimulated) ASC for nerve regeneration. Microphotographs showing the β-Tubulin III+ axons for various experimental groups: Autograft; empty fibrin-hydrogel nerve conduit "FNC"; FNC's wall loaded with unstimulated ASC, i.e., intramural ASC delivery "FNC-W(ASC)"; FNC's lumen loaded with unstimulated ASC, i.e., intraluminal ASC delivery "FNC-L(ASC)"; FNC's wall loaded with NGF-stimulated ASC, i.e., intramural NGF-ASC delivery "FNC-W(NGF-ASC)"; FNC's lumen loaded with NGF-stimulated ASC, i.e., intraluminal NGF-ASC delivery "FNC-L(NGF-ASC)". The blue staining is Hoechst indicating cell nuclei. The scale bar represents 100 µm.

In contrast to the distinct axonal growth response (Figure 4 and 5) found for the various stimuli conditions, SC exhibited no differences (Figure S2A,B). However, the molecular differences for the recruited SC in the animals due to the various stimuli conditions remain to be investigated. We hypothesize that the SC recruited in response to the various stimuli conditions may vary in their phenotype (sensory vs. motor) and secretome.

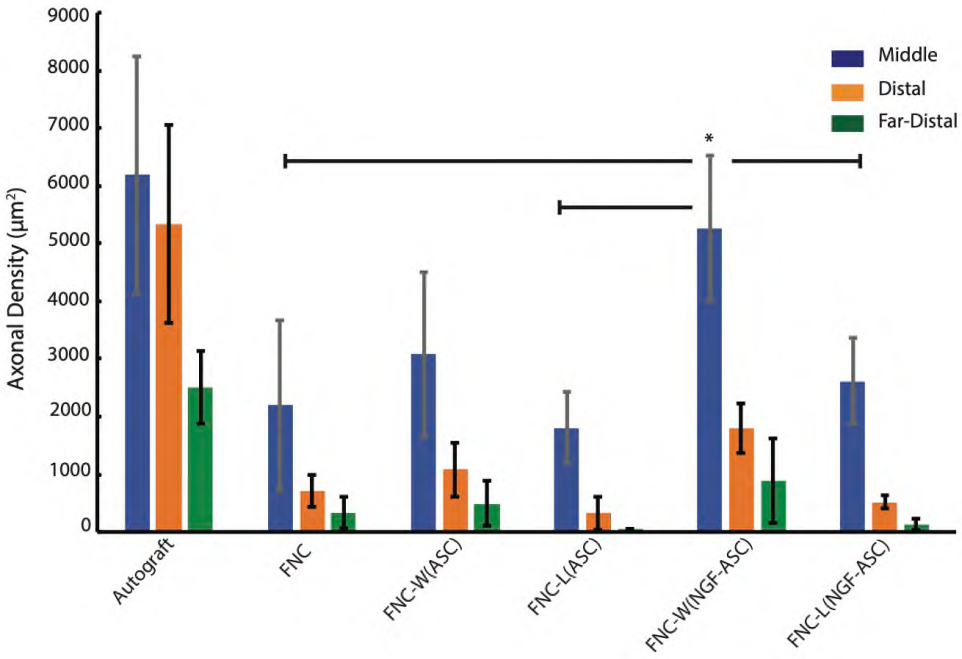

Figure 5. Fibrin assisted delivery of (stimulated) ASC for nerve regeneration. Quantitative measurements of β-Tubulin III+ axons for various experimental groups: Autograft; empty fibrin-hydrogel nerve conduit "FNC"; FNC's wall loaded with unstimulated ASC, i.e., intramural ASC delivery "FNC-W(ASC)"; FNC's lumen loaded with unstimulated ASC, i.e., intraluminal ASC delivery "FNC-L(ASC)"; FNC's wall loaded with NGF-stimulated ASC, i.e., intramural NGF-ASC delivery "FNC-W(NGF-ASC)"; FNC's lumen loaded with NGF-stimulated ASC, i.e., intraluminal NGF-ASC delivery "FNC-L(NGF-ASC)". The bars represent the mean ± SD of n = 6. Significant differences at * $p < 0.05$ are indicated in comparison to all other experimental groups.

4. DISCUSSION

We hypothesized impactful change in the ability of ASC in response to growth factors' stimuli for supporting the axonal regeneration. Therefore, the present study was designed to investigate the growth promoting capacity of ASC following NGF- or VEGF-stimulation in vitro and to further translate in vitro findings into animals in combination with two different cell delivery approaches, i.e., FNC assisted intramural vs. intraluminal ASC loading for studying the early nerve regeneration. In the present study, experiments were conducted using NGF- or VEGF-stimulated ASC. A large portion of the research investigating the neurotrophic effect of ASC involved SCLC derived from ASC while studies on undifferentiated ASC remained scarce [8,82]. Although an SCLC phenotype seems to be desirable for optimal regenerative support, 2 to 4 weeks of in vitro differentiation process represents a major obstacle for the clinical use of SCLC. On the other hand, the therapeutic benefits of SCLC derived from ASC vs. undifferentiated ASC are still not clear. Nevertheless, several studies suggest a similar therapeutic potential for both cell types [104–106]. There are two mechanisms explaining the regenerative potency of undifferentiated ASC. The first hypothesis states that ASC might undergo an in vivo trans-differentiation in response to the regenerative microenvironment [102,105,107,108]. The other hypothesis postulates that the neurotrophic potential of ASC may lie in the secretome containing a wide range of biochemical and molecular factors [109]. ASC exosomes releasing miRNA21, miRNA222 and

miRNAlet7a play an important role in neuronal survival by inhibiting apoptotic pathways. In addition, the secretome of ASC contains nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-ß), and platelet-derived growth factors (PDGF) [98,101,133–135]. Furthermore, transplanted ASC may enhance the recruitment of endogenous SC to the injury site [104].

To the factors secreted by ASC, adult neurons may respond differently in contrast to embryonic neurons, as the trophic dependency of the various subsets of neurons in the peripheral nervous system may change with the age. However, several studies have shown that injury-induced expression of various growth factors and surface receptors are responsive for both adult and embryonic neurons [74,136] . Thus, the embryonic DRG assay used in our study should provide relevant information on the neurotrophic capacity of the conditioned medium derived from ASC.

The data obtained in the present study clearly showed the enhanced potency of ASC in response to a NGF-stimulus on axonal regeneration. The CM derived from ASC after 72 h of NGF-stimulation (i.e., CM-NGF-ASC), as well as the CM of unstimulated ASC supplemented with exogenous NGF (i.e., CM-ASC+NGF) induced significant axonal outgrowth. Furthermore, there is no significant difference found in the axonal outgrowth promoted by either of these culture conditions indicating the growth-promoting function of NGF in combination with ASC's CM. However, the exact mechanism of the underlying molecular functions needs to be determined along with the analysis of the secretome profile of ASC resulting from various experimental stimuli. On the contrary, VEGF culture conditions did not result in enhanced axonal regeneration in vitro. These observations may indicate no direct effect of VEGF in vitro on axonal regeneration. Interestingly, ASC's secretome contains a wide range of growth factors, in addition to high amounts of VEGF (i.e., 2000 to 3000 pg/mL) [134,135] indicating that there may be no need for additional exogenous VEGF administration for ASC therapies. Thus, the in vitro results and data instructed the design of animal experiments using single factor NGF-stimulated ASC.

In agreement with our in vitro findings, NGF-stimulated ASC exhibited a potential for promoting enhanced early nerve regeneration as evidenced by the increased number of β-Tubulin III+ axons. The functional restoration of human nerve injuries is critical and

controlled by the timely entry of regenerative axons into the distal nerve segment, which in turn is influenced by the quality (i.e., axonal growth speed) and quantity (axonal number) of the early axonal regeneration. Thus, the present study reports on the important aspects of early nerve regeneration in the context of ASC-fibrin-hydrogel based therapeutic conduits.

In the middle of the fibrin conduit, i.e., regenerated nerve tissue, the density of regenerating axons for the animals treated with intramural delivery of NGF-stimulated ASC was statistically comparable to the autograft treatment, indicating not only the benefits of stimulated stem cells but also the importance of the cell delivery route. However, the outcome in the subsequent distal and far-distal segments was significantly higher for the autograft group. Further on, the intramural delivery of NGF-stimulated ASC, i.e., W(NGF-ASC) promoted better axonal regeneration as evidenced by the increased axonal number in the different parts of the conduit including the far-distal segment, although the axonal growth was found in the distal part of the conduit in all the other groups. The beneficial effects of delivering individual VEGF for nerve regeneration are unclear, given the number of studies reporting variable outcomes, i.e., increased angiogenesis with or without functional benefits [137,138]. On the other hand, studies reporting on sequential release of VEGF and NGF exhibited beneficial effects. However, the early axonal regeneration in those animals appeared poorer than in autograft animals as evidenced by significantly lower axonal regeneration in the middle graft after 4 weeks [139]. Therefore, the regeneration levels achieved in our present study appeared superior to that of dual-factor NGF and VEGF treatment, indicating the potential of the NGF-stimulated ASC for better nerve regeneration and for reducing the complexity of administering additional exogenous growth factors.

In order to improve the outcomes, i.e., suboptimal results obtained particularly at the distal part of the nerve, we would need to further refine our technique by optimizing the loading density of stem cells and by incorporating topographical guidance structures. Given the focus i.e., early nerve regeneration of the present study, we only measured early axonal regeneration. However, the data and knowledge obtained in the present study create a strong rationale for further evaluations of functional restoration, i.e., behavioral recovery, electrophysiological recovery and re-innervation in a long-term study.

The delivery of cells within a conduit is a subject of research and the most optimal delivery system remains to be determined. Particularly for FNC, only a few methods have been evaluated separately and no systematic comparison was reported so far. In general, the

fibrin solution loaded with cells can be injected into the lumen of the FNC [115]. An alternative approach is to integrate the fibrin solution loaded with cells into the main structure of the FNC's wall by polymerization [116]. The least interesting way is to suspend the cells in a carrier medium and to place them into the lumen of the conduit [117], which usually results in leakage of cells.

In our study, we tested the first two approaches by delivering the NGF-stimulated ASC either by FNC assisted intramural or by intraluminal loading. For this, fibrin conduits measuring 14 mm in length, 1 mm in wall thickness and 2 mm in lumen were produced for bridging a 10 mm nerve gap-injury. It is widely accepted that fibrin hydrogels possess a macro porous structure with a pore size of 10–20 µm [140]. Therefore, the conduits used in our study may possess pore sizes in the range of 2–4 µm and 10–20 µm respectively for intramural and intraluminal fibrin structures that can naturally facilitate the diffusion of ASC secretome. ASC based exosomes and microvesicles are in the range of a sub-micrometer size (i.e., 150 nm to 200 nm) [109,141]. Thus, the macro porous fibrin hydrogels enable easy passage of nanometer-sized vesicles and related soluble factors. However, the density of transplanted cells within the hydrogel microenvironment may account for different outcomes achieved through different delivery routes in the present study. In the case of the intramural delivery system, 2 million cells were loaded in 600 µL of carrier fibrin hydrogel in contrast to the intraluminal delivery system where 2 million cells were loaded in 40 µL of carrier hydrogel. Although homogeneity of the cell treatment (i.e., the dose of the total cells) for animals was ensured, the high density of cells within the lumen of the fibrin nerve conduit may result in high concentrations of local growth factors. Thus, the highly enriched luminal microenvironment of the intraluminal delivery system may impede the speed of axonal regeneration in contrast to the intramural delivery system. Taken together, the underlying structural and cellular factors may explain appropriately the better outcome achieved by the intramural cell delivery system in the present study.

Fibrinogen concentrations used in the present study for both intramural and intraluminal constructs were based on our earlier research work and no-detrimental effects were found when the fibrin matrix was used alone. As we previously reported [104], ASC showed survival signals up to 14 days after transplantation in NC. These findings indicate that the regenerative effects of transplanted ASC are mediated by the initial boost of released growth factors. The cell delivery route dependent outcome observed in our present study

may partly be attributed to the potency of the transplanted cells. Further investigations on the spatiotemporal profile of the transplanted ASC may provide important understanding and knowledge for the optimization of ASC therapies. The present study was conducted using rat-derived ASC, therefore further studies are required using human ASC, in order to translate these findings into clinical settings.

5. CONCLUSION

We report on a growth factor based strategy for the ex-vivo stimulation of ASC and show the evidence for the enhanced neurotrophic potency of ASC in response to NGF-stimulation, but not VEGF, to promote axonal regeneration in vitro and in vivo. Furthermore, our study reveals the importance of a fibrin-hydrogel conduit assisted intramural delivery system in improving the therapeutic efficacy of ASC for nerve regeneration. Together, these findings provide new knowledge and important insights for the development of ASC-based therapies for treating nerve gap-injuries. Further studies are required in order to assess the long-term impact of ex-vivo stimulated ASC on the anatomical and functional nerve regeneration, and for the understanding of the secretome-profile of ASC resulting from various stimuli-conditions.

Supplementary Materials: The following are available online at www.mdpi.com/2306-5354/7/2/42/s1, Figure S1: Stem cell characterization, Figure S2: (A) Immunostaining of Schwann cell growth. (B) Quantitative analysis of Schwann cell growth.

Author Contributions: S.M. contributed conception and design of the study; S.M. supervised the project. K.P. and L.D. conducted both in vitro and in vivo experiments; S.M., D.K., R.G., D.S. and K.P. contributed for important intellectual interpretation of the study; S.M. and K.P. performed the statistical analysis; K.P. wrote the first draft of the manuscript; S.M. revised the manuscript. All the authors contributed to manuscript revision, read and approved the manuscript submission.

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Disclosure: Authors declare no conflicts of interest.

Supplementary figures

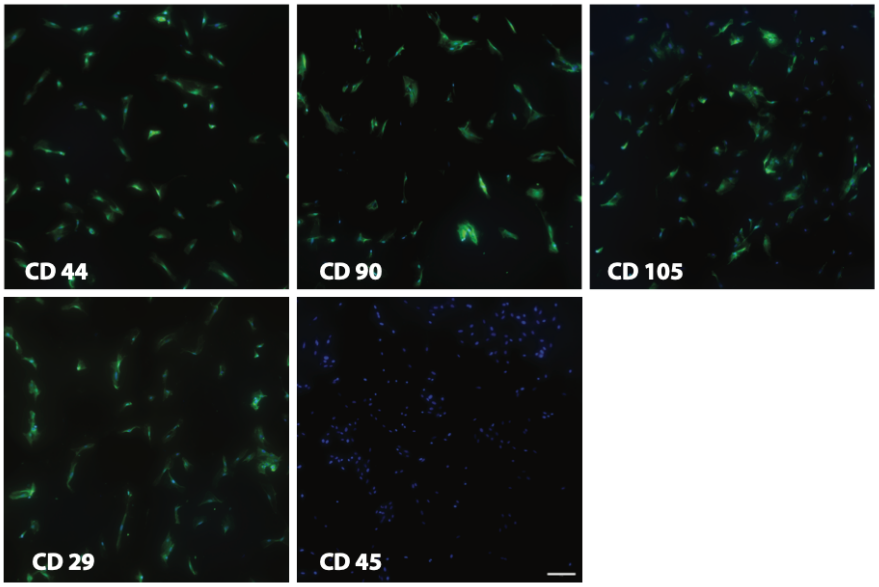

Figure S1. Stem cell characterization. Immunostaining and characterization of rat adipose stem cells (ASC) that are positve for mesenchymal stem cell markers CD44, CD 90, CD105 and CD29 in green. ASC are negative for hematopoietic marker CD45. The blue staining is Hoechst indicating cell nuclei. The scale bar represents 100 µm.

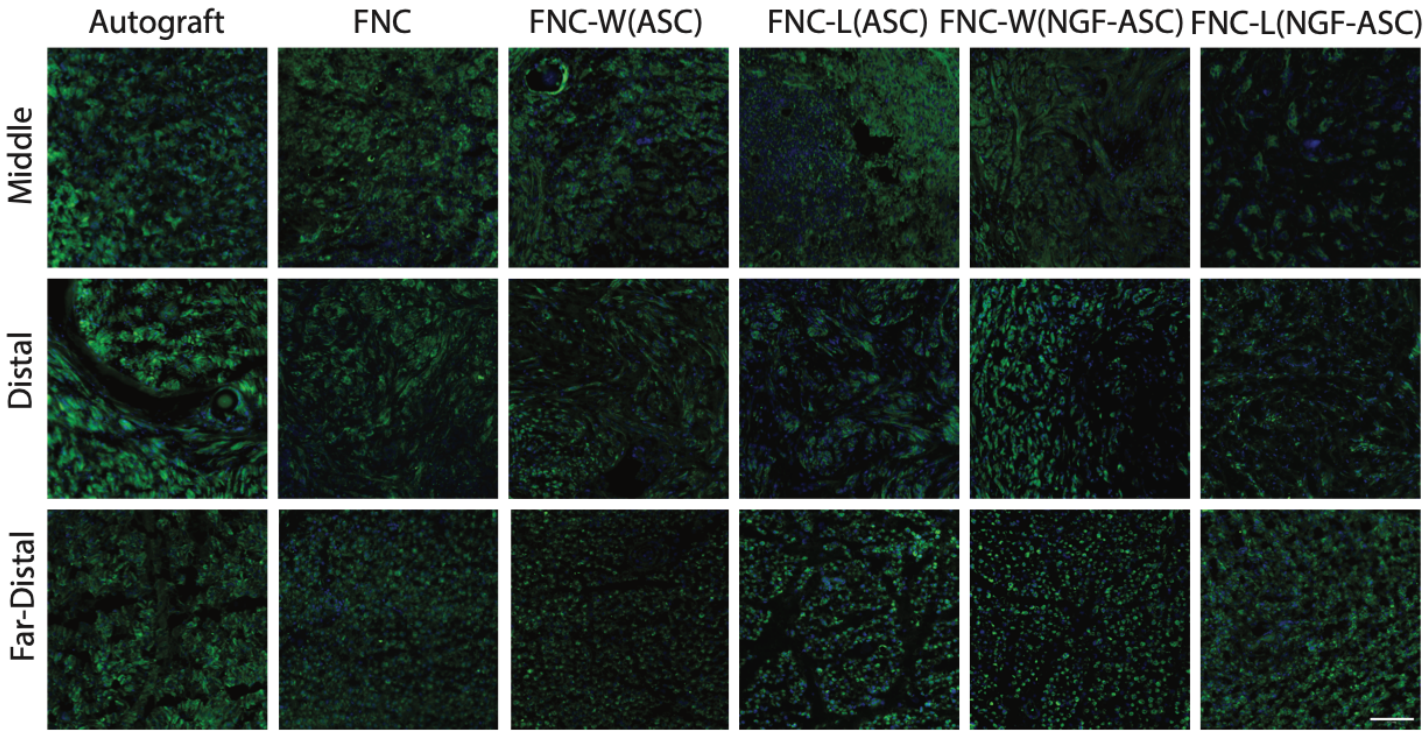

Figure S2A. Immunostaining of Schwann cell growth. Microphotographs showing the S100+ Schwann cell structures for the experimental groups: Autograft; empty fibrin-hydrogel nerve con- duit “FNC”; FNC’s wall loaded with unstimulated ASC, i.e., intramural ASC delivery “FNC-W(ASC)”; FNC’s lumen loaded with unstimulated ASC, i.e., intralumen ASC delivery “FNC-L(ASC)”; FNC’s wall loaded with NGF-stimulated ASC, i.e., intramural NGF-ASC delivery “FNC-W(NGF-ASC)” and FNC’s lumen loaded with NGF-stimulated ASC, i.e., intraluminal NGF-ASC delivery “FNC-L(NGF-ASC)”. The blue color is Hoechst indicating cell nuclei. The scale bar represents 100 µm.

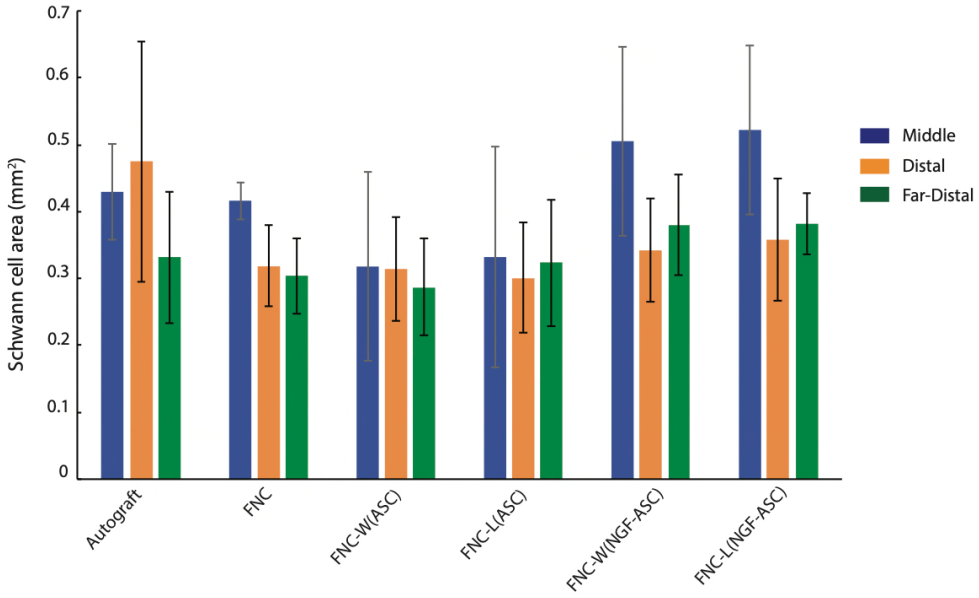

Figure S2B. Quantitative analysis of Schwann cell growth. Measurement of the S100+ Schwann cell structures for the experimental groups: Autograft; empty fibrin-hydrogel nerve conduit "FNC"; FNC's wall loaded with unstimulated ASC, i.e., intramural ASC delivery "FNC-W(ASC)"; FNC's lumen loaded with unstimulated ASC, i.e., intralumen ASC delivery "FNC-L(ASC)"; FNC's wall loaded with NGF-stimulated ASC, i.e., intramural NGF-ASC delivery "FNC-W(NGF-ASC)" and FNC's lumen loaded with NGF-stimulated ASC, i.e., intraluminal NGF-ASC delivery "FNC-L(NGF-ASC)". The bars represent the mean ± SD of n=6.

MANUSCRIPT 2

Modulation of Human Adipose Stem Cells' Neurotrophic Capacity Using a Variety of Growth Factors for Neural Tissue Engineering Applications: Axonal Growth, Transcriptional and Phosphoproteomic Analyses In Vitro

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ABSTRACT

We report on a potential strategy involving the exogenous neurotrophic factors (NTF) for enhancing the neurotrophic capacity of human adipose stem cells (ASC) in vitro. For this, ASC were stimulated for three days using NTF, i.e., nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), NT4, glial cell-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF). The resulting conditioned medium (CM) as well as individual NTF exhibited distinct effects on axonal outgrowth from dorsal root ganglion (DRG) explants. In particular, CM derived from NT3-stimulated ASC (CM-NT3-ASC) promoted robust axonal outgrowth. Subsequent transcriptional analysis of DRG cultures in response to CM-NT3-ASC displayed significant upregulation of STAT-3 and GAP-43. In addition, phosphoproteomic analysis of NT3-stimulated ASC revealed significant changes in the phosphorylation state of different proteins that are involved in cytokine release, growth factors signaling, stem cell maintenance, and differentiation. Furthermore, DRG cultures treated with CM-NT3-ASC exhibited significant changes in the phosphorylation levels of proteins involved in tubulin and actin cytoskeletal pathways, which are crucial for axonal growth and elongation. Thus, the results obtained at the transcriptional, proteomic, and cellular level reveal significant changes in the neurotrophic capacity of ASC following NT3 stimulation and provide new options for improving the axonal growth-promoting potential of ASC in vitro.

Keywords: human adipose stem cells; neurotrophic factors; peripheral nerve injuries; axonal regeneration; Schwann cells; ex vivo stimulation; dorsal root ganglion

1. INTRODUCTION

Peripheral nerve injuries with loss of functional tissue are a common clinical problem whereby patients suffer lifelong sensory-motor deficits in spite of advanced microsurgical procedures and functional follow-ups [1,5,9,11]. However, nerve injuries have no viable alternative to match the regenerative level of the gold standard (autologous nerve grafts) [1,11,45]. Thus, the need for nerve tissue engineering approaches emerged.

The process of timely and effective nerve regeneration is primarily influenced by the regenerative microenvironment, which comprises sustained growth factor release, permissive extracellular matrix scaffolds, and Schwann cells facilitating the axonal guidance [22,142]. Therefore, the unsatisfactory clinical outcomes obtained so far can be attributed to the failed maintenance of a permissive microenvironment within the niche of the regenerating nerve tissue. Nerve tissue engineering strategies aim at sustaining an effective microenvironment for functional nerve regeneration [1,11,26]. Within this context, Schwann cells (SC) play a key role [47].

Following injury, SC initiate Wallerian degeneration, express a wide range of cytokines and neurotrophic factors (NTF), and support axonal path finding [25,81,143]. SC express p75, TrkA, TrkB, TrkC, and GFRα receptors [144–151], which are responsive for various growth factors and rapidly establish paracrine signaling in response to endogenous growth factors [25,26,81,152]. Several studies proved the beneficial effects of transplanted SC for nerve repair and axonal regeneration [48,75,83,153]. However, the harvest of autologous SC requires a painful nerve biopsy for the patient, followed by rather challenging culture conditions, which result in significant treatment delay [84,85]. Therefore, stem cells gained a growing interest as a viable alternative [154,155].

Adipose-derived stem cells (ASC) can be harvested in abundance from adipose tissue [91–93], grow fast, and have a low immunogenic profile [96,97]. They secrete an array of neurotrophic factors, chemokines, and miRNA [98–101] and can be differentiated into SC-like cells (SCLC) in order to improve their neurotrophic properties [98,99,101,102]. ASC differentiation into SCLC requires extended stimulation involving a complex array of exogenous factors over up to three weeks, indicating a major hurdle to their clinical use. Moreover, it was demonstrated that SCLC rapidly revert to fibroblast cell-like characteristics after the withdrawal of the differentiation medium. Therefore, their beneficial effects

remain questionable [20,104–106], and there is a clear need for developing simple and effective strategies for enriching the neurotrophic properties of ASC [8].

Following injury, a wide range of NTF, which can be attributed to neuronal and non-neuronal sources [23], are rapidly overexpressed [7,22,69]. Thus, the therapeutic targets of severed nerves, i.e., neurons and glial cells, require neurotrophic support and structural guidance for repair and regeneration [156]. Although the peripheral nervous system is capable of spontaneous regeneration due to the intrinsic regenerative capacity, long-gap and chronic nerve injuries, in particular, require exogenous trophic and topographical support for effective regeneration [72]. However, recent studies showed the deleterious effects of using large number of biological factors for promoting axonal regrowth while the therapeutic benefits are being ineffective [157]. Thus, the need for effective and viable alternatives such as cell therapies emerged [155]. Cell with improved trophic capacity for addressing the complex needs of severed nerves would circumvent the side effects associated with application of large number of exogenous factors [154]. ASC and SCLC were shown to support nerve regeneration by releasing a variety of biological factors with mitotic and neuritogenic activity, although neurotrophic capacity of ASC is still inferior to SC [158]. Growth factors and related receptors play key role in the SC lineage and their autocrine survival loop [159] [52]. Interestingly, ASC were found to express most of the receptors for NTF [98,100,103,160–162] suggesting that ASC may operate like SC in response to NTF stimulation. Within this context, we recently demonstrated enhanced neurotrophic capacity of ASC in response to nerve growth factor (NGF) stimulation in vitro and in vivo [158].

Given these facts, we hypothesized that NTF may have a fundamental role in the neurotrophic capacity of the stem cells, which is related to SC lineage, which in turn is an important topic to basic neurobiology (SC lineage) and nerve tissue engineering. Therefore, it is fundamental to understand the role of variety of NTF individually on the fate of ASC at cellular and molecular levels. However, studies elucidating the influence of NTF on ASC biology (neurotrophic capacity) are largely missing, thus there is a great need for shedding light on this key topic [158].

Therefore, in the present study, we have evaluated different NTF representing neurotrophins, i.e., NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and NT4; the glial cell-derived neurotrophic factor (GDNF) family of ligands, i.e., GDNF, and neurotrophic cytokines, i.e., CNTF [22,69,70] for ex vivo stimulation of ASC. Subsequently,

we assessed the axonal growth-promoting capacity of a conditioned medium (CM) derived from NTF-stimulated ASC as well as individual NTF and found significant effects on axonal outgrowth. Furthermore, molecular changes taking place in the stimulated ASC as well as in the treated DRG explant cultures were studied by transcriptional and phosphoproteomic analyses.

2. MATERIALS AND METHODS

2.1. Isolation and Culture of Human Adipose Stem Cells (ASC)

Adipose tissue was obtained from healthy patients undergoing elective liposuction or abdominoplasty. All patients had signed an informed consent form prior to the surgery. The isolation of ASC was performed under sterile conditions according to a well-established protocol [102]. The fat tissue was cleaned from erythrocytes by rinsing with 0.01 M phosphate-buffered solution (PBS) and centrifugation, further it was minced and digested with 0.1% (w/v) Type I Collagenase (Gibco Life Technologies, Cat. No. 17100017) for 3 h at 37 °C and centrifuged for 5 min at 1500 rpm and 4 °C. The resulting pellet was re-suspended in growth medium (GM) i.e., Dulbecco`s Modified Eagle`s Medium (DMEM, Gibco, Cat. No. 41965039) supplemented with 10% Foetal Bovine Serum (FBS) (PAN-Biotech, EU-approved, Cat. No. P40-47500) and 1% Penicillin/Streptomycin (BioConcept, Cat. No. 4-01F00-H). Extracted ASC were seeded at a density of 3000 cells/cm2 and cultured at 37 °C with 5% CO2 in a humid atmosphere with GM being changed every 72 h. Cell passage was performed at 90% confluence by using 0.25% Trypsin-EDTA (BioConcept, Cat. No. 5-51F00- H) and resulting cells at passage 2 (P2) or 3 (P3) were used for the experiments.

2.2. Human ASC Characterization

P2 human ASC were seeded on 24-well plates for characterization. Cells were fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 10 min and permeabilized and blocked in PBS containing 0.1% Triton X-100 and 1% normal goat serum (NGS) (i.e., dilution buffer) for 20 min at RT. ASCs were incubated overnight at 4 °C with the human mesenchymal stromal cell markers monoclonal mouse anti-CD44 (1:1000), monoclonal mouse anti-CD90 (1:200), monoclonal mouse CD105 (1:200), monoclonal rabbit anti-CD29 (1:100) and polyclonal rabbit anti-CD45 (1:500) (Abcam, Cat. No. ab93758). Cells were then washed in PBS and incubated for 1 h at RT with the secondary antibody goat anti-mouse

Alexa Fluor 488 (1:500, Abcam, Cat. No. ab150109) and goat anti-rabbit Alexa Fluor 488 (1:500, Abcam, Cat. No. ab150061), and Hoechst 33258 nuclear staining (1:1000, Sigma Aldrich, Cat. No. 94403). Subsequently, digital images were acquired at 20x magnification (numerical aperture 0.45) by using a Nikon Eclipse Ti2 inverted fluorescent microscope (Nikon Eclipse Ti2-E, -E/B, Nikon Corporation, Japan) and a Nikon DS-Qi2 camera (Nikon Corporation, Japan). The images were automatically stitched by the Nikon NIS-Elements AR image analysis software (NIS- Elements AR Analysis 5.11.00 64-bit, Laboratory Imaging, spol. s.r.o., Czech Republic). Results are shown in Figure S1.

2.3. Explantation of Chicken Embryonic Dorsal Root Ganglions (DRG)

Fertilized chicken eggs were ordered from Gepro Geflügelzucht AG (Flawil, Switzerland). The eggs were shipped at ambient temperature and incubated at 37.8 ± 0.2 °C under 100% relative humidity for 10 days (E10). After incubation, the eggs were wiped with 70% ethanol and embryos were collected under a laminar airflow cabinet. Dissection of the E10 embryos was performed with the help of a stereomicroscope following a standard dissection protocol [154,163,164]. DRG were explanted from the lumbar part of the spine and stored in GM on ice until cell culture.

2.4. Quantitative Measurement of NT3 by ELISA

CM medium from the NT-3 stimulated ASC and unstimulated ASC was collected. ELISA kit was obtained from R&D systems (Cat. No. dy267 and DY008) and analysis was run as per the protocol provided by manufacturer. Briefly, 96-well plates were incubated with 100 µL of the respective capture antibody overnight at 4 49C. Plates were then blocked for a minimum of 1 h at RT by adding 300 µL of reagent diluent to each well. Subsequently, 100 µL of sample or standards were added per well and incubated 2 h at RT. Standards were prepared using serial dilutions. Plates then underwent consecutive incubations at RT for each 20 min, with 100 µL of Streptavidin-HRP A and with 100 µL of Substrate Solution. Finally, 50 µL of Stop Solution was added to each well. Optical density of each well was determined immediately, using the Synergy H1 micro-plate reader (BioTek Instruments, Winooski, VT, USA) set to 450 nm. For wavelength correction, readings at 540 nm were subtracted from the readings at 450 nm. The program used was Gen5 2.05 (BioTek Instruments, Winooski, VT, USA).

2.5. Experimental Design for the Axonal Outgrowth Assay

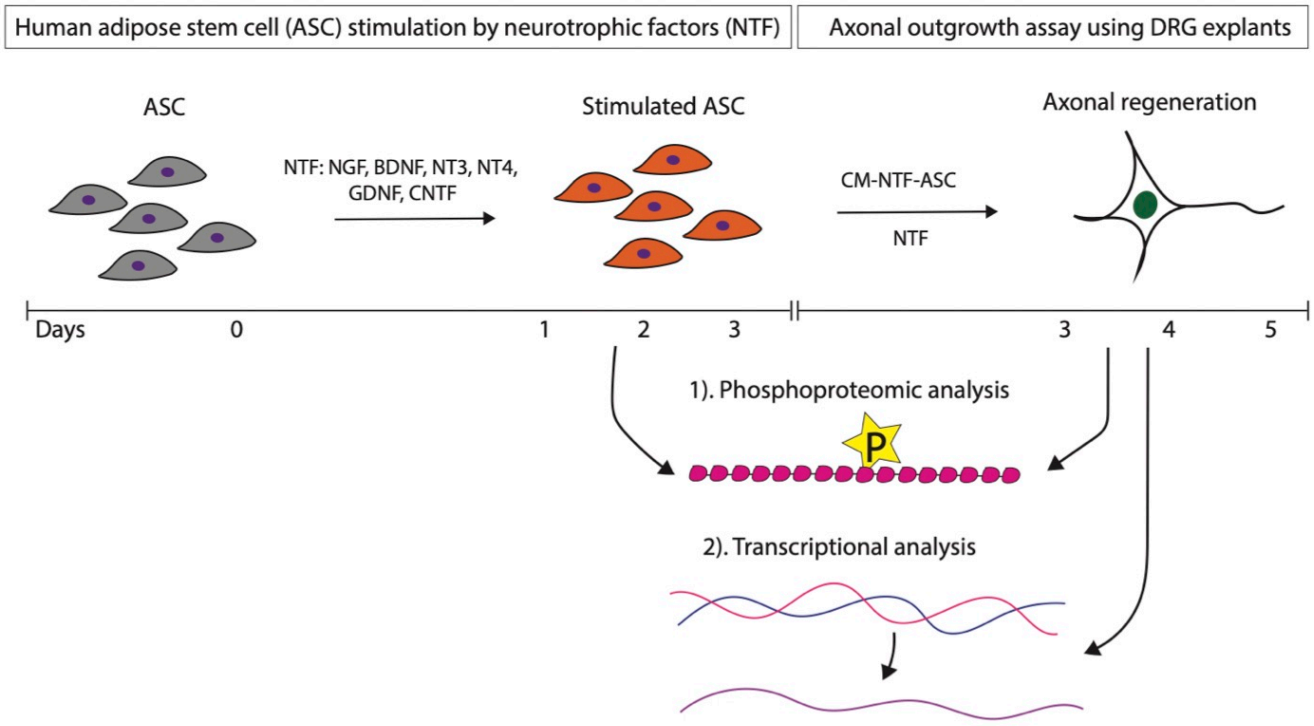


Figure 1. Ex-vivo stimulation of adipose stem cells using different neurotrophic factors. Key. ASC: adipose stem cells; NTF: neurotrophic factors; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor; NT3: neurotrophin 3; NT4: neurotrophin 4; GDNF: glial cell-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; CM-NTF-ASC: conditioned medium derived from NTF-stimulated ASC; DRG: dorsal root ganglion.

As illustrated in Figure 1, freshly splitted ASC seeded at 50 - 60% confluency in 24 well plates with 500 µl of volume were stimulated either by rhNGF (NGF-ASC), rhGDNF (GDNF-ASC), rhBDNF (BDNF-ASC), rhCNTF (CNTF-ASC), rhNT3 (NT3-ASC) or rhNT4 (NT4-ASC) or without neurotrophic factor (ASC) for 72 h. Resulting conditioned medium (CM), i.e., CM-NGF-ASC, CM-GDNF-ASC, CM-BDNF-ASC, CM-CNTF-ASC, CM-NT3-ASC, CM-NT4-ASC and CM-ASC was subsequently used for the DRG assay. As controls, culture conditions with growth media supplemented with the respective NTF, i.e. NGF alone (NGF), GDNF alone (GDNF), BDNF alone, (BDNF), CNTF alone (CNTF), NT3 alone (NT3), NT4 alone (NT4), and the growth medium without NTF-supplementation (GM) were used. For all conditions neurotrophic factors were applied at 10ng/ml based on preliminary experiments [74]. Neurotrophic factors were purchased from R&D Systems Inc., Minneapolis, USA (Art. No. 256-GF, 212-GD, 248-BDB, 257-NT, 267-N3, 268-N4). For the axonal outgrowth assay, DRG-explants were cultured at a density of one per well onto 24-well plates in 500 µl of the respective condition. Cultures were maintained in a humid atmosphere at 37 °C and 5% CO2 for 48 h and images

were captured at 5x and 10x magnification. In total 3 independent experiments resulting in a total of 12 DRG-explant cultures for each experimental condition were performed.

2.6. Immunocytochemistry of DRG Cultures

After 48 h, bright field images with a phase contrast of DRG-explants were taken at 5x magnification using a Zeiss Axio Vert.A1 inverted fluorescent microscope (Carl Zeiss AG, Germany) and a Zeiss AxioCam MRc camera (Carl Zeiss AG, Germany). DRG-explants were then fixed in 4% PFA at RT for 10 min and permeabilized and blocked in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA) (i.e., dilution buffer) for 20 min at RT. For immunocytochemistry, the cultures were incubated overnight at 4 °C with the primary antibody monoclonal mouse anti-β-Tubulin III (1:1000, Sigma-Aldrich, Cat. No. T8578) for regenerating axons. The cultures were then washed in PBS and incubated for 1 h at RT with the secondary antibody sheep anti-mouse Cy3 (1:500, Sigma Aldrich, Cat. No. C2181) and Hoechst 33258 nuclear staining (1:1000, Sigma Aldrich, Cat. No. 94403). Digital images of the stained specimens were acquired at 15x magnification (numerical aperture 0.45) using the Nikon Eclipse Ti2 inverted fluorescent microscope and a Photometrics prime 95B 25mm camera (Teledyne photometrics, Arizona, USA). The images were automatically stitched by the Nikon NIS-Elements AR image analysis software.

2.7. Quantitative Analysis of Axonal Outgrowth

DRG-explant cultures were measured for axonal length and axonal area with an automated and standardized analysis mask created by using the Nikon NIS- Elements AR image analysis software.

2.8. Quantitative RT-PCR for Regeneration Associated Genes (RAG) in DRG

DRG-explants grown for 48 h in NT3-stimulated ASC's conditioned medium (CM-NT3-ASC), unstimulated ASC's conditioned medium (CM-ASC), NT3-supplemented GM (NT3) and GM only (GM) were evaluated for up-regulation of the regeneration associated genes STAT-3 and GAP-43, β-Actin was used as housekeeping gene. A total of 500 ng RNA per sample was converted into cDNA following the protocol for the SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen™, Cat. No. 11766050) and using the SimpliAmp™ Thermocycler (Applied Biosystems™ A24811 by life technologies, Thermo Fisher Scientific

AG, Switzerland). Amplification was performed with the QuantStudio™ 3 Real-Time PCR Systems (applied biosystems, Thermo Fisher Scientific AG, Switzerland) using Fast Start Universal SYBR Green Master Mix (Roche, Cat. No. 04 913 850 001). 20 ng of cDNA were used per well. The primers used are listed in Figure 4B and were designed for the specie gallus gallus. All reactions were performed using the same conditions: 50 °C for 2 min, 95 °C for 10 min; 40 cycles at: 95 °C for 15 s, 60 °C for 1 min. The experiment was repeated 3 times and all experimental samples were assayed in triplicate. A negative control (RNase-free water) was always included. Expression of STAT-3 and GAP-43 were determined in relation to β-actin RNA levels.

2.9. Phosphoproteomic Analysis

Freshly splitted ASC seeded at 50 – 60% confluency in 75 cm^2 culture flasks were either stimulated by NT3 (NT3-ASC) or no growth factor (ASC) for 72 h. After incubation, the cells were detached by scratching and subjected to 2 cycles of centrifugation for 5 min at 1500 rpm and 4 °C and PBS washing. After the last wash, the PBS was discarded and the cell pellet was immediately transferred to 80 °C for storage. For the DRG samples the same experimental groups and culture conditions as for the qRT-PCR experiment were applied. After 48 h of incubation in the respective conditioned medium, the DRG-explants were subjected to the same treatment as described above for ASC, except of centrifugation which was performed at 900 rpm. A protein yield of at least 100 µg per experimental condition was required for phosphoproteomics. The experiments were repeated 3 times, resulting in 3 independent samples for ASC and DRG-explants.

Cells were lysed in 8M Urea (Sigma), 0.1M ammonium bicarbonate in the presence of phosphatase inhibitors (Sigma P5726&P0044) using strong ultra-sonication (Bioruptor, 10 cycles, 30 seconds on/off, Diagenode, Belgium). Protein concentration was determined by a BCA assay (Thermo Fisher Scientific) using a small sample aliquot. 250 µg of proteins were digested as described previously (PMID:27345528), reduced with 5 mM TCEP for 60 min at 37 °C and alkylated with 10 mM chloroacetamide for 30 min at 37 °C. After diluting the samples with 100 mM ammonium bicarbonate buffer to a final urea concentration of 1.6 M, the proteins were digested by incubation with sequencing-grade modified trypsin (1/50, w/w; Promega, Madison, Wisconsin) overnight at 37 °C. After acidification using 5% TFA, the

peptides were desalted on C18 reversed-phase spin columns according to the manufacturer's instructions (Macrospin, Harvard Apparatus) and dried under vacuum.

Peptide samples were enriched for phosphorylated peptides using Fe(III)-IMAC cartridges on an AssayMAP Bravo platform as recently described (PMID: 28107008). The setup of the µRPLC-MS system was as described previously (PMID:27345528). Chromatographic separation of peptides was carried out using an EASY nano-LC 1200 system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 µm x 37 cm) packed in-house with 1.9 µm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Aliquots of 1 µg total peptides were analyzed per LC-MS/MS run using a linear gradient ranging from 95% solvent A (0.1% formic acid) and 5% solvent B (80% acetonitrile, 19.9% water, 0.1% formic acid) to 30% solvent B over 90 minutes at a flow rate of 200 nl/min. Mass spectrometry analysis was performed on a Tribrid Orbitrap Lumos mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each MS1 scan was followed by high-collision-dissociation (HCD) of the 10 most abundant precursor ions with dynamic exclusion for 30 seconds. The total cycle time was approximately 1 s. For MS1, 1e6 ions were accumulated in the Orbitrap cell over a maximum time of 100 ms and scanned at a resolution of 120,000 FWHM (at 200 m/z). MS2 scans were acquired at a target setting of 100%, accumulation time of 54 ms and a resolution of 30,000 FWHM (at 200 m/z). Singly charged ions and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 30%, the mass isolation window was set to 1.4 m/z and one microscan was acquired for each spectrum.

The acquired raw-files were imported into the Progenesis QI software (v2.0, Nonlinear Dynamics Limited), which was used to extract peptide precursor ion intensities across all samples applying the default parameters. The generated mgf-files were searched against a human/chicken database containing usually observed contaminants and a total of 41592 (human, download date 2019/03/07 from www.uniprot.org)/55856 (chicken, download date 2020/04/14 from www.uniprot.org) protein sequences using MASCOT and the following search criteria: full tryptic specificity was required (cleavage after lysine or arginine residues, unless followed by proline); 3 missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) and phosphorylation (STY) were applied as variable modifications; mass tolerance of 10 ppm (precursor) and 0.02 Da (fragments). The database search results were filtered using the ion score to set the false

discovery rate (FDR) to 1% on the peptide and protein level, respectively, based on the number of reverse protein sequence hits in the datasets. The relative quantitative data obtained were normalized and statistically analyzed using our in-house script as above (PMID:27345528). The complete list of quantified phosphorylation sites is provided as supplemental data. All raw data associated with this manuscript are publicly available (data availability).

2.10. Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [PubMed ID: 30395289] partner repository with the dataset identifier PXD019015 and 10.6019/PXD019015.

2.11. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) following Bonferroni procedure with post hoc multiple comparisons using SPSS (version 15.0; SPSS, Chicago, IL, USA). Values with $P < 0.05$ were considered significant.

3. RESULTS

3.1. Human ASC Characterization

The phenotype of ASC was quantitatively characterized using representative images and ImageJ for analysis. The cells were found to be positive for mesenchymal marker CD29 at 87%, CD44 at 88%, CD90 at 92%, and CD105 at 93%, and negative for hematopoietic marker CD45 (Figure S1). Further, NT3-stimulated ASC also displayed similar expression pattern of mesenchymal stem cell (MSC) markers including S100 of SC at 96%, but no expression was observed for other specific markers, i.e., GFAP and p75 (Figure S2).

3.2. Distinct Effects of NTF on Axonal Outgrowth

As illustrated in Figure 1, various NTF were used for stimulation of axonal growth. Interestingly, all the growth factors promoted considerable axonal outgrowth in comparison to GM. Notably, NT3 promoted significant axonal outgrowth (Figure 2A-C). Quantitative measurements of axonal length (in μm) from DRG-explants treated with growth factors

resulted in 413 ± 182 for NGF, 405 ± 116 for GDNF, 419 ± 73 for BDNF, 352 ± 74 for CNTF, 463 ± 121 for NT3, 291 ± 51 for NT4 and 282 ± 41 for GM (Table 1). Interestingly, axonal growth pattern in response to various NTF treatments appeared to be distinctive. NGF promoted dense axonal growth without longer projections in contrast to GDNF, which resulted in relatively longer axonal projections without showing branching effect. BDNF influenced axonal elongation as well as branching. Notably, NT3 promoted radially aligned axonal growth with relatively longer axonal projections. NT4 as well as CNTF resulted in only minimal axonal outgrowth (Figure 2). Quantitative measurements of axonal area (in mm^2) in DRG cultures amounted to 1.903 ± 1.239 for NGF, 1.252 ± 0.486 for GDNF, 1.566 ± 0.479 for BDNF, 0.766 ± 0.286 for CNTF, 1.819 ± 0.700 for NT3, 0.674 ± 0.123 for NT4 and 0.616 ± 0.125 for GM (Table 1).

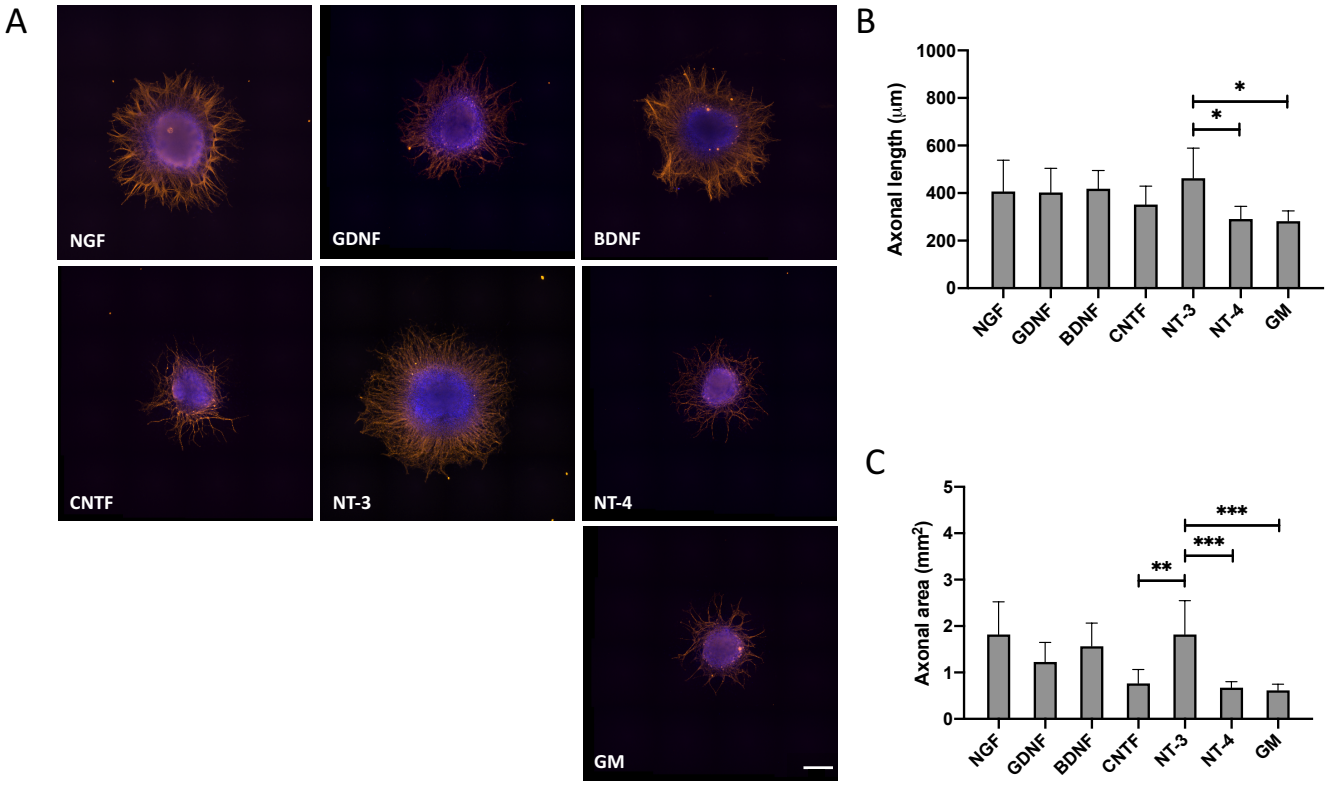


Figure 2. Distinct effects of individual NTF on axonal outgrowth. **(A)** Microphotographs of dorsal root ganglion (DRG) explants treated with NTF or growth medium alone (GM), (**B**) Quantitative measurements of axonal length (µm), and (**C**) Measurements of axonal area (mm^2). The scale bar represents 500 µm. The bars represent mean ± SD of $n = 12$. Significant differences are indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Group	Axonal length (μm)	Axonal area (mm^2)
NGF	413 ± 182	1.903 ± 1.239
GDNF	405 ± 116	1.252 ± 0.486
BDNF	419 ± 73	1.566 ± 0.479
CNTF	352 ± 74	0.766 ± 0.286
NT-3	463 ± 121	1.819 ± 0.700
NT-4	291 ± 51	0.674 ± 0.123
GM	282 ± 41	0.616 ± 0.125
CM-ASC	469 ± 99	1.166 ± 0.456
CM-NGF-ASC	526 ± 87	2.460 ± 0.586
CM-GDNF-ASC	505 ± 75	1.512 ± 0.334
CM-BDNF-ASC	598 ± 118	1.918 ± 0.547
CM-CNTF-ASC	599 ± 58	2.032 ± 0.643
CM-NT3-ASC	765 ± 134	3.423 ± 0.798
CM-NT4-ASC	522 ± 80	1.617 ± 0.427

Table 1. Quantitative measurements of the axonal outgrowth resulting from DRG explants treated with differential stimuli. Data expressed in mean ± SD of *n* = 12.

3.3. Conditioned Medium Derived from Stimulated ASC

NTF-stimulated ASC's conditioned medium (CM) resulted in an enhanced axonal outgrowth in comparison to unstimulated ASC and NTF treatment alone (Figure 3A - C). Quantitative measurements of axonal length (in μm) amounted to 526 ± 87 for CM-NGF-ASC, 505 ± 75 for CM-GDNF-ASC, 598 ± 118 for CM-BDNF-ASC, 599 ± 58 for CM-CNTF-ASC, 765 ± 134 for CM-NT3-ASC, 522 ± 80 for CM-NT4-ASC and 469 ± 99 for CM-ASC (Table 1). Significant differences were observed for CM-BDNF-ASC ($p<0.05$), CM-CNTF-ASC ($p<0.001$), CM-NT3-ASC ($p<0.001$) and CM-NT4-ASC ($p<0.001$). Particularly, CM-NT3-ASC triggered significantly robust axonal outgrowth in comparison to all other treatment conditions. Quantitative measurements of axonal area (in mm^2) resulted in 2.460 ± 0.586 for CM-NGF-ASC, 1.512 ± 0.334 for CM-GDNF-ASC, 1.918 ± 0.547 for CM-BDNF-ASC, 2.032 ± 0.643 for CM-CNTF-ASC, 3.423 ± 0.798 for CM-NT3-ASC, 1.617 ± 0.427 for CM-NT4-ASC and 1.166 ± 0.456 for CM-ASC (Table 1). In agreement with the axonal length measurements, CM-NT3-ASC exhibited significantly higher axonal outgrowth area (Figure 3C). Notably, CM-NT3-ASC promoted numerous radially aligned axons displaying longer projections than all other conditions (Figure 3A).

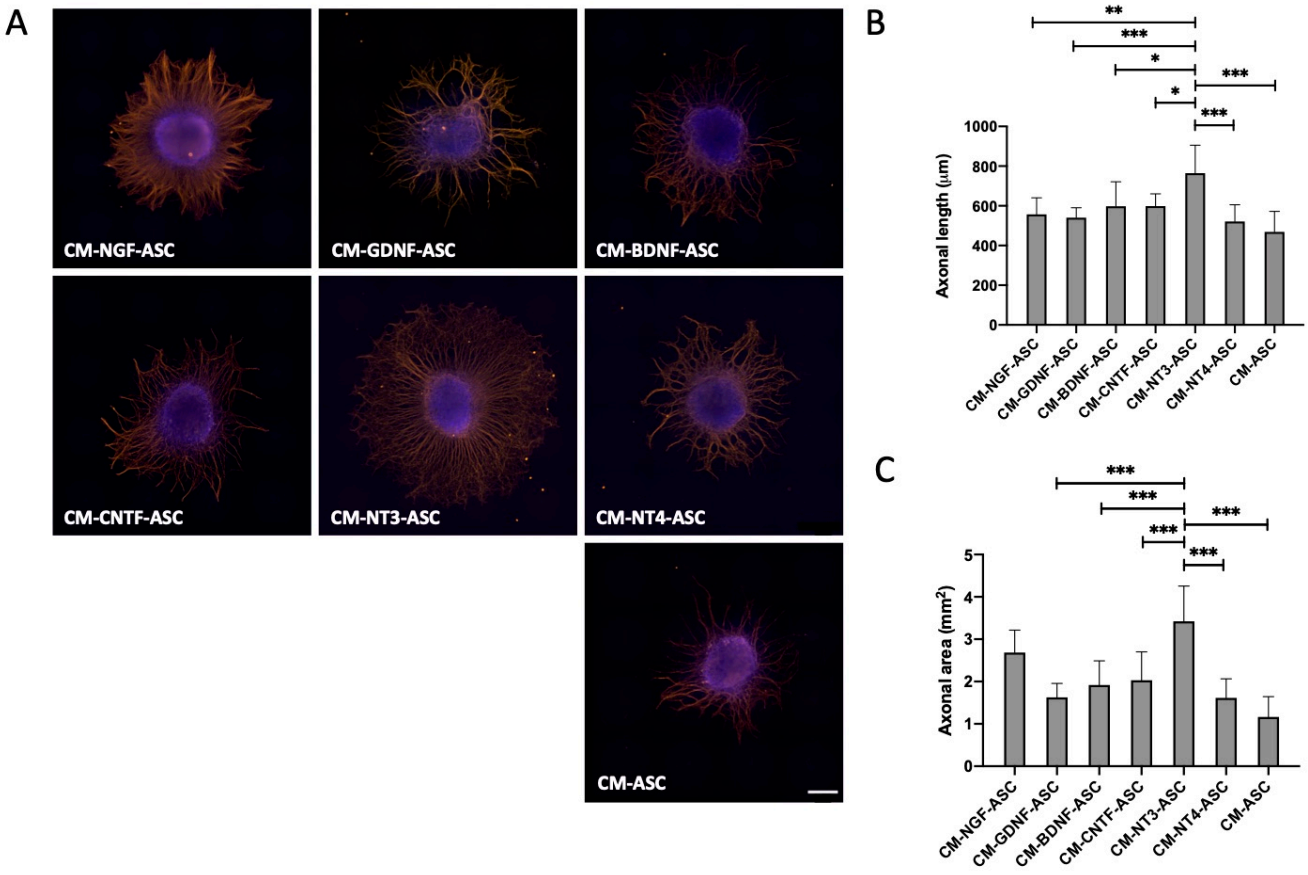


Figure 3. Neurotrophic potency of conditioned medium (CM) derived from NTF-stimulated ASC for supporting axonal outgrowth. **(A)** Microphotographs of dorsal root ganglion (DRG) explants treated with CM derived from NTF-stimulated ASC, (**B**) Quantitative measurements of axonal length (µm), and (**C**) Measurements of axonal area (mm^2). The scale bar represents 500 µm. The bars represent mean ± SD of $n = 12$. Significant differences are indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

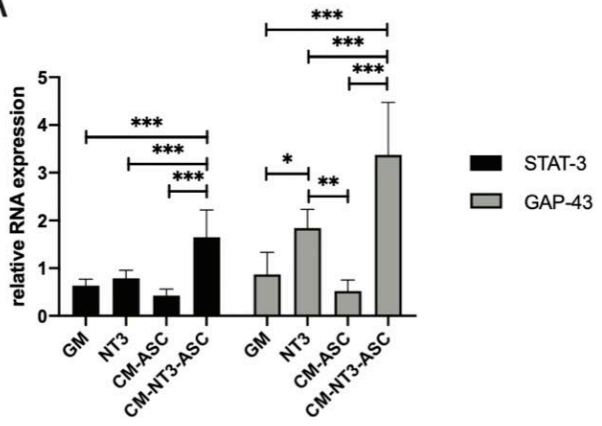
3.4. NT3 content

Quantitative analysis of the CM resulting from NT3-stimulated ASC amounted to 1.1 ± 0.32 ng/mL, which is significantly higher than physiological concentration 25–40 pg/mL [165–167] and lower than the amount 10 ng/mL used for the experiment. In contrast, unstimulated ASC showed no detectable NT3.

3.5. Transcriptional Analysis of RAG in Treated DRG-Explants

Analysis by qRT-PCR for relative STAT-3 RNA levels resulted in Ct values of 1.65 ± 0.57 for CM-NT3-ASC, 0.42 ± 0.14 for CM-ASC, 0.79 ± 0.17 for NT3 and 0.63 ± 0.14 for GM (Figure 4C). Relative GAP-43 Ct RNA values showed 3.37 ± 1.10 for CM-NT3-ASC, 0.52 ± 0.23 for CM-ASC, 1.84 ± 0.39 for NT3 and 0.87 ± 0.46 for GM. Significant upregulation of the regeneration associated genes STAT-3 and GAP-43 was observed for DRG-explants treated with CM-NT3-ASC in comparison to all other conditions (Figure 4A-C).

A



B

Gene	Forward Primer	Reverse Primer
STAT-3	5'-TGC CTA TTA GCC TCC GAT TG-3'	5'-CCA GAC ACT GGC AGT CAA GA-3'
GAP-43	5'-CTC ACG TCT GTG AGC CTG TC-3'	5'-TGA CTG GGA TTT TTC CTG CT-3'
Actin-β	5'-TGC GCA TAA AAC AAG ACG AG-3'	5'-GAC TGC TGC TGA CAC CTT CA-3'

C

Condition	STAT-3	GAP-43
GM	0.63 ± 0.14	0.87 ± 0.46
NT3	0.79 ± 0.17	1.84 ± 0.39
CM-ASC	0.42 ± 0.14	0.52 ± 0.23
CM-NT3-ASC	1.65 ± 0.57	3.37 ± 1.10

Figure 4. Transcriptional analysis of DRG explants treated with different stimuli. **(A)** Analysis of STAT-3 and GAP-43 mRNA expression by qRT-PCR, **(B)** Primer sequences used for reverse transcription with polymerase chain reaction, and **(C)** Quantitative measurements of mRNA expression. Expression values are relative to β-actin expression and are therefore in arbitrary units, shown as the mean ± SD of $n = 9$. Significant differences are indicated * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.6. Quantitative Phosphoproteomic Analysis

To obtain a global view on the signaling events taking place upon growth factor treatment a mass spectrometry-based phosphoproteomic analysis was performed. Two different experiments were carried out comparing NT3-ASC vs. ASC as well as DRG-explant cultures treated with various stimuli conditions, i.e., CM-NT3-ASC, CM-ASC, NT3, and GM. All quantitative analyses were performed in biological triplicates analyzing phosphopeptide-enriched samples using a label-free quantification of LC-MS approach (PMID: 30867597). In total, 10352 and 7440 phosphorylation sites were quantified for ASC and DRG-explants, respectively (Figure 5, Figures S3 and S4, and Tables S1 and S2). ASC in response to NT3 stimulation exhibited 21 significantly changing phosphorylation sites (Figure 5A). These include FRMD8 and NOTCH2 which are involved in the regulated growth factors release and neural progenitor cell (NPC) maintenance and differentiation [168–170]. Enrichment analysis of the altered phosphorylation sites further showed that the notch signaling pathway and the active domain of notch2 were significantly affected by the NT3-stimulation (Figure 5B, Figure S3, and Table S1).

Subsequent comparison of DRG-explants treated with CM-NT3-ASC vs. GM displayed 10 significantly changing phosphorylation sites (Figure 5C and D, Figures S3 and Table S2). Proteins associated with these sites include TBCEL, EVL, SPEN and MAP1A which are linked to growth cone formation and the polymerization, reorganization and stabilization of axonal cytoskeletal proteins (i.e., tubulin and actin) [171–178]. An enrichment analysis further

confirmed that the tubulin- and actin-cytoskeletal pathway was significantly altered between these two conditions (Figure 5D), with elevated activity found for CM-NT3-ASC. The control (GM) DRG-explants were also compared to other treatment conditions, i.e., CM-ASC and NT3 (Figure S4); we found no robust changes. Most changes in the phosphoproteome were observed upon CM-NT3-ASC whereas treatment with NT3 alone or CM-ASC did not have a significant impact on the *p*-value frequency distribution (Figure S3A – C and Figure S4), indicating that the CM of NT3-stimulated ASC is the trigger for the phosphorylation-based signaling events identified. Interestingly, these findings are consistent with the axonal outgrowth studies (Figure 3).

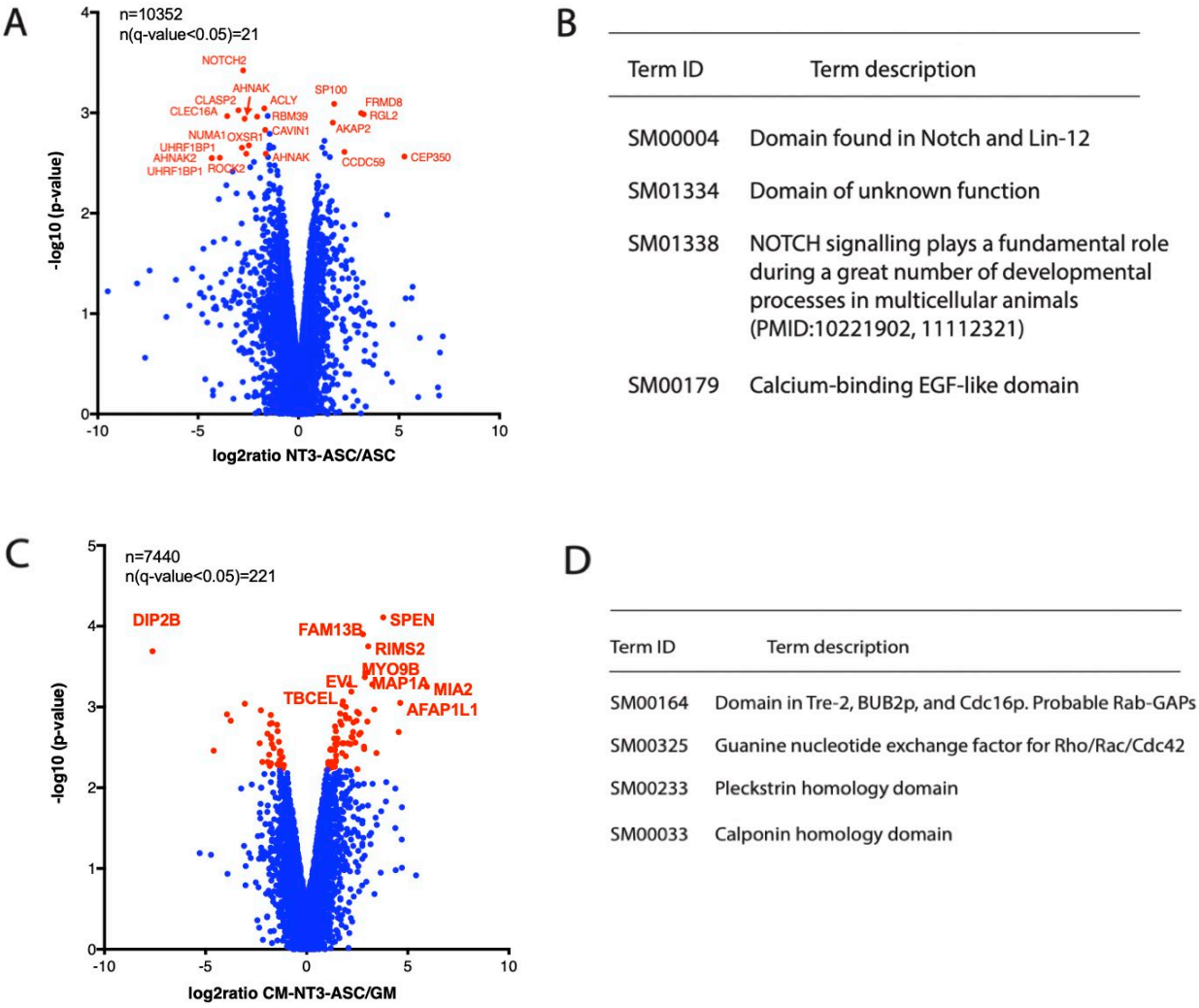


Figure 5. Quantitative phosphoproteomics analysis. **(A)** Volcano plots of phosphorylation site ratios and significance (*p*-value) obtained for NT3-stimulated ASC and unstimulated ASC comparisons. Significant hits with a *q*-value of less than 0.05 as computed by SafeQuant using paired t-test and Benjamini-Hochberg correction are indicated in red. The proteins associated with the 21 most significantly changing phosphorylation sites are also shown, **(B)** Significantly enriched terms ($p < 0.05$) found for the most significant hits using STRING, **(C)** Volcano plots of phosphorylation site ratios and significance (*p*-value) obtained for CM-NT3-ASC and GM comparisons. Significant hits with a *q*-value of less than 0.05 as computed by SafeQuant using paired t-test and Benjamini-Hochberg correction are indicated in red. The proteins associated with the 10 most significantly changing phosphorylation sites are also shown and **(D)** Significantly enriched terms ($p < 0.05$) found for the most significant hits using STRING.

4. DISCUSSION

Functional nerve regeneration is clinically challenging [45]. After injury, affected neurons and glial cells require neurotrophic support and structural guidance for survival and regeneration [72]. Peripheral nervous system is capable of spontaneous regeneration due to the intrinsic regenerative capacity. However, gap injuries and chronic nerve injuries, in particular, require exogenous trophic and topographical support for effective regeneration [2,179]. Nerve tissue engineering holds promise for developing a viable new strategy subjective to the availability of effective neurotrophic cell sources [180]. MSC derived from variety of tissues have been widely known to possess multilineage capacity and multifunctional potency [100,155,158,180]. The ability of these cells to support the trophic needs of the neuronal cells for survival, development, and plasticity can be defined as "neurotrophic capacity" within the context of functional multipotency of the stem cells. ASC release a variety of neurotrophins and support neuronal survival and axonal outgrowth, suggesting their neurotrophic activity [100,180]. Furthermore, several studies reported the potential of ASC for supporting the neuronal survival and axonal regeneration by releasing a variety of growth factors, cytokines, extracellular matrix (ECM) molecules, and miRNAs [109] . Thus, the potential use of ASC for treating neurological disorders and injuries gained significant clinical interest, although the trophic capacity of ASC still remained inferior to SC [158,180].

The neurotrophic potential of ASC lies within the secretome containing a variety of biochemical and molecular factors [8,109,154,155,162]. ASC exosomes with miRNA21, miRNA222, and miRNAlet7a support neuronal survival by inhibiting apoptotic pathways. In addition, the secretome of ASC contains wide range of growth factors, i.e., nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), and platelet-derived growth factors (PDGF) [98,101,102,109,133–135]. Furthermore, transplanted ASC may activate the endogenous SCs, resulting in enhanced recruitment to the injury site [93].

Within this context, we hypothesized important changes in the neurotrophic capacity of ASC in response to exogenous NTF stimulation. Therefore, the present study was designed to test the neurotrophic capacity of ASC following exogenous NTF stimulation. For this, we have

primarily evaluated NTFs representing three different families, i.e., the neurotrophins (NGF, BDNF, NT3, and NT4), the GDNF family of ligands (GDNF), and the neurotrophic cytokines (CNTF), for ex vivo stimulation of ASC. The resulting condition medium (CM) as well as individual NTFs were investigated separately for their capacity to promote axonal outgrowth using DRG explants in vitro.

The cellular and molecular biology of the different species are highly conserved [181,182] and further, the structural homology of the various growth factors between human and chicken is more than 80% [183,184]. Moreover, several studies have demonstrated effective axonal outgrowth from chick DRG explants in response to human NTFs [74]. The limitation of DRG model is that it only considers sensory neurons. DRG explants possess many advantages such as easy accessibility, handling, modest costs, and three-dimensional cellular contact between neuronal and non-neuronal cells. Although the injury-induced changes are largely common among various stages of the neurons, embryonic neurons possess higher plasticity than the adult neurons [74,158,163,164,179]. However, several studies have shown that injury-induced expression of various growth factors and surface receptors that are responsive for both adult and embryonic neurons [100,136]. Thus, our in vitro model involving biological materials of different species should provide relevant information on the neurotrophic capacity of the secretome derived from ASC after differential stimuli.

To build a scientific rationale using a multimodal system, it is extremely important to consider the compatibility of the study design with the associated principles of concerned disciplines, i.e., stem cell biology, neurobiology, pharmacology, and toxicology. For this, there were set of guidelines, i.e., non-clinical safety evaluation of drug and biologic combinations published by European Medical Agency (EMA) as well as food and drug administration (FDA) [185]. In compliance with the above-indicated guidelines, our multimodal system represents a valuable in vitro model for basic and translational studies.

NGF is a well-studied neurotrophic factor due to its role in neuronal survival and axonal regeneration. In our assay, NGF clearly elicited abundant axonal outgrowth with strong branching, as reported earlier [74]. This effect is caused by NGF's specific binding to the high-affinity TrkA receptor of sensory and sympathetic neurons [186]. GDNF, through its binding to the high-affinity GFRα complex [147], promoted relatively longer axonal projections in contrast to NGF. GDNF has been proven to exert trophic effects on injured motor and sensory axons [77]. This observation is supported by its upregulation in the

ventral and dorsal root following injury [187]. The diverse functions of BDNF are mainly mediated by the TrkB receptor [23], and it was found to be especially elevated in sensory nerves after injury [188]. BDNF, interestingly, promoted axonal elongation as well as branching, as reported earlier [151,189–192]. In our assay, NT3 appeared to be effective for triggering axonal outgrowth, with significantly longer axonal projections. This observation is supported by previous reports, where its administration in vitro prevented sensory neuronal loss [75,193]. NT3 exerts its action by binding to the high-affinity receptor TrkC. CNTF and NT4 showed minimal enhancement of axonal outgrowth in terms of axonal length as well as axonal area, which is comparable to DRG explants cultured in growth medium alone. Godinho et al. [75] evidenced low axonal density for CNTF grafts in vivo through binding to CNTFRα receptors. Downregulation of CNTF expression after nerve injury [194] may indicate its less pronounced role in regeneration. NT4, on the other hand, binds to the TrkB receptor in motoneurons [195] and specifically improves the functional reinnervation of slow motor units [196].

Interestingly, all NTF used for the ex vivo stem cell stimulation resulted in improved trophic potency of ASC for promoting axonal regeneration. In particular, NT3-stimulated ASC (CM-NT3-ASC) in comparison to all other NTF, elicited robust axonal outgrowth. When compared to NGF, it becomes evident that the increased axonal area did not mainly originate from axonal branching, since NT3-induced axonal projections are linearly aligned and well organized, but most probably from increased neuronal survival. The role of NT3 in preventing neuronal loss following injury has been well documented [75,193,197,198]. ASC have been shown to express receptors for various NTF [101,103,160–162]. Thus, the exogenous NTF stimuli may amplify the neurotrophic capacity of ASC through their receptor binding, leading to enhanced expression of regeneration-associated molecules, cytokines, and growth factors. Interestingly, a phosphoproteomic analysis revealed significant changes taking place in the phosphorylation of different proteins involved in growth factor signaling, cytokine release, stem cell population maintenance, and differentiation. In particular, FRMD8 and Notch2 showed significantly enhanced phosphorylation in NT3-stimulated ASC in comparison to unstimulated ASC.

FRMD8 mediates the controlled release of growth factors and cytokines from cells by regulating the active complex of ADAM17 and iRhom [170]. Lack of FRMD8 activity leads to degradation of ADAM17 and iRhom, resulting in low or no release of the growth factors

[170]. Several growth factors, i.e., EGF receptor ligand amphiregulin (AREG), transforming growth factors alpha (TGFα), heparin-binding EGF, epigen, and epiregulin, are regulated by the coordinated function of these molecules [199,200]. Within this context, elevated phosphorylation of FRMD8 in the NT3-stimulated ASC gains significance and shows the possibility of the increased release of growth factors and cytokines. Therefore, a detailed analysis of the composition of the resulting secretome may provide more insights into the topic.

Notch2 plays a key role in the maintenance of neural progenitor cells (NPC) and differentiation [168]. Several studies reported that the mere activation of notch2 is sufficient for the maintenance and proliferation of the NPC [201,202]. On the other hand, a lack of Notch2 expression leads to differentiation of NPC into the neuronal phenotype [168]. These observations suggest that NT3-stimulated ASC might have attained endogenous changes toward neuronal lineage, but not complete differentiation. These findings are further strengthened by microscopic evidence showing the NT3-stimulated ASCs' transition into S100 positive phenotype within 3 days, although these cells did not yet express other typical glial markers such as GFAP and p75. Furthermore, DRG explant treated with CM-NT3-ASC also exhibited significant changes taking place in the phosphorylation of different proteins involved in the actin and tubulin cytoskeletal pathways. In particular, EVL, SPEN, RIMS2 and MAP1A exhibited significantly enhanced phosphorylation in the DRG treated with CM-NT3-ASC compared to all other controls.

EVL regulates actin filament polymerization and controls a range of processes that are dependent on the actin cytoskeleton remodeling, e.g., axonal growth and extension [99]. An actin-filled growth cone precisely navigates axonal elongation, involving a dynamic process of actin polymerization and depolymerization by continuous sampling of guidance cues within the microenvironment [177]. Furthermore, netrin-1-induced filopodia formation was found to be EVL-dependent and directly correlated with EVL phosphorylation at a regulatory PKA site [173,176]. Thus, EVL proteins, a conserved family of actin-regulatory proteins, are essential for growth cone formation, dynamics of axonal growth, and elongation in response to differential guidance cues. On the other hand, MAP1A is a major component of the microtubule-linked fibrillar matrix of axons [175], regulating the microtubule cytoskeletal organization, which is crucial for axonal growth and activity-dependent remodeling of the axonal projections [175]. Within this context, SPEN is required for plasticity of the neurons,

axonal outgrowth, and branching [178]. RIMS2 is known to positively regulate the dendrite arborization.

Taken together, these findings and observations at the phosphoproteomic level support the notion of enhanced trophic capacity (axonal growth) of ASC in response to NT3-stimulation.

To further analyze the molecular changes taking place in DRG explants treated with CM derived from NTF-stimulated ASC, a transcriptional analysis of the regeneration-associated genes (RAG) STAT-3 and GAP-43 was performed using qRT-PCR. For this, DRG explants treated with the most promising treatment condition, i.e., CM-NT3-ASC, were processed. The resulting data revealed a significant upregulation of RAGs. GAP-43 is upregulated in the perikaryon of neuronal cells after axotomy-induced inflammatory environment, calcium influx, and retrograde signaling of neurotrophic factors [9,22,26]. GAP-43 participates in the formation of the axonal growth cone and its associated lamellipodiae and filopodiae for axon elongation and path finding [7,23]. GAP-43 is also upregulated in denervated SC, where it supports their survival and maintenance [9,203,204]. Furthermore, GAP-43 is involved in supporting the long-term survival of denervated SC, as well as in the maintenance of the phenotype of repair SC [152]. JAK/STAT3, on the other hand, belongs to the main transcriptional mediators that encode for the expression of growth-associated genes such as GAP-43 [9,19,152]. Its transcription is activated by receptor-ligand binding and has been shown to play an important role in the initiation phase of axonal outgrowth and early regeneration response [205]. Our results illustrate the correlation between activation of the STAT-3 transcriptional pathway and upregulation of the effector RAG, i.e., GAP-43 in DRG explants treated with CM-NT3-ASC. A possible explanation for the upregulation of the STAT-3 pathway in DRG explants might be the array of growth-promoting factors within the secretome of NT3-stimulated ASC.

Therapeutic targets of damaged peripheral nerve, various subsets of neurons and glial cells require molecular, trophic, and guidance support for effective regeneration. Although peripheral nervous system is capable of spontaneous regeneration due to the intrinsic regenerative capacity, long-gap and chronic nerve injuries, in particular, require exogenous bioactives support for regeneration [72]. Clinically optimal nerve regeneration can be achieved by orchestrating a permissive microenvironment at the injury site. For this, complex array of biological factors is required [72,156]. Up-to-date, autologous nerve grafting remains the gold standard treatment for nerve gap injuries, which might be

attributed to the presence of Schwann cells and structural extracellular matrix proteins [25,45]. For the future, nerve conduits combined with a cell-based therapy might be the most natural and effective way to mimic the autograft's features [206]. Neurotrophic cells provide long-term availability of NTF in a physiological context, which includes adequate release kinetics and quantities, and feedback mechanisms regulating NTF release [24]. Moreover, transplanted stem cells create an interactive regenerative niche, not only by secreting factors but also by responding to endogenous signals. Although the results are promising, existing strategies for improving the trophic capacity of ASC, i.e., biochemical induction [86,102] or genetic induction [75,207,208], are associated with several drawbacks such as lengthy processes (more than three weeks), safety and regulatory hurdles. Moreover, several studies reported the deleterious effects of using large number of biological factors for axonal outgrowth in vivo [157]. Thus, there is need for simple yet effective strategies such as cell with improved trophic capacity that is similar to SC.

We, therefore, propose in the present study a simple and effective strategy involving ex vivo stimulation of ASC using exogenous NTF. Notably, our approach may reduce or avoid the complexity of administering additional exogenous growth factors in vivo, while maximizing the regenerative support for severed neuro-glial cells. Among all the NTF, NT3 exerted a remarkable stimulus to enhance the neurotrophic capacity of ASC, as evidenced by the phenotypic transition, axonal outgrowth, transcriptional, and phosphoproteomic data. NT3 quantitative measurements resulting from CM of different experiments together with the axonal outgrowth outcome indicate that ASC's enhanced neurotrophic capacity for triggering axonal growth in vitro is NT3 dependent. Although the underlying mechanism remains to be elusive, several lines of evidence at cellular and molecular level support the endogenous changes that are linked with neuro-glial lineage of ASC after NT3-stimulation. Within this context, detailed account of composition of the secretome derived from the NT3-stimulated ASC would hold a key for further insights.

Several studies indicate that SC are the source of the NT3 in the damaged peripheral nerves, but not the axons, as evidenced by the NT3 heterozygous knockout mice displaying the decreased SCs, myelin retardation and high neuro-filament packing density of axons [159,209–211]. Further, NT3 is a potent factor for sensory neuronal survival and axonal regeneration [178]. Moreover, NT3 is crucial for survival of SCs following injury and mature SCs in adult nerves are able to survive in the absence of axons by establishing an autocrine

survival loop [159]. Thus, the NT3 appears to be one of the key factors of the autocrine loop that supports long-term SC survival [159,209], nerve regeneration, and remyelination after peripheral nerve injury [209]. However, chronic nerve injuries result in SC atrophy and reduced expression of receptors and growth factors by SC [210,211]. Given these facts, NT3 cross-talk with DRG neurons appears to be one-way.

Thus, the data and knowledge obtained in the present study forms strong basis for the further refinement of the therapeutic strategy and evaluation in animals (immune-deficient nude rats), i.e., tracking/analysis of transplanted cells, anatomical, behavioral, electrophysiological recovery, and re-innervation.

5. CONCLUSION

In the present study, we demonstrated the feasibility of modulating ASC's neurotrophic potential in vitro as evidenced by axonal outgrowth, transcriptional, and phosphoproteomic data. In particular, the conditioned medium resulting from NT3-stimulated ASC triggered robust axonal outgrowth with significantly longer projections. Together, these findings and observations provide new knowledge that should be relevant to ASC biology, basic neurobiology, and nerve repair. Further, these findings need to be validated using adult neurons and translated into a rat model of nerve-gap injury for evaluating the biology of the NT3-ASC and spatiotemporal function.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/9/1939/ s1, Figure S1: Characterization of human adipose-derived stem cells by immunocytochemistry, Figure S2: Characterization of NT3-stimulated human adipose-derived stem cells by immunocytochemistry, Figure S3: Frequency plots of *p*-values obtained for DRG explants with different treatment conditions compared to control GM, Figure S4: Quantitative phosphoproteomic analysis, Table S1: Phosphoproteomic data obtained from stimulated-ASC and unstimulated-ASC, and Table S2: Phosphoproteomic data obtained from DRG explants treated with differential stimuli.

Authors contribution statement: S.M. designed the research and supervised the project; K.M.P., A.S., and V.P. conducted the experiments; S.M., D.F.K., R.G., D.J.S., A.S., and K.M.P. organized the database; S.M. and K.M.P. performed the statistical analysis; K.M.P. wrote the first draft of the manuscript; S.M. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Disclosure: Authors declare no conflicts of interest.

Supplementary figures

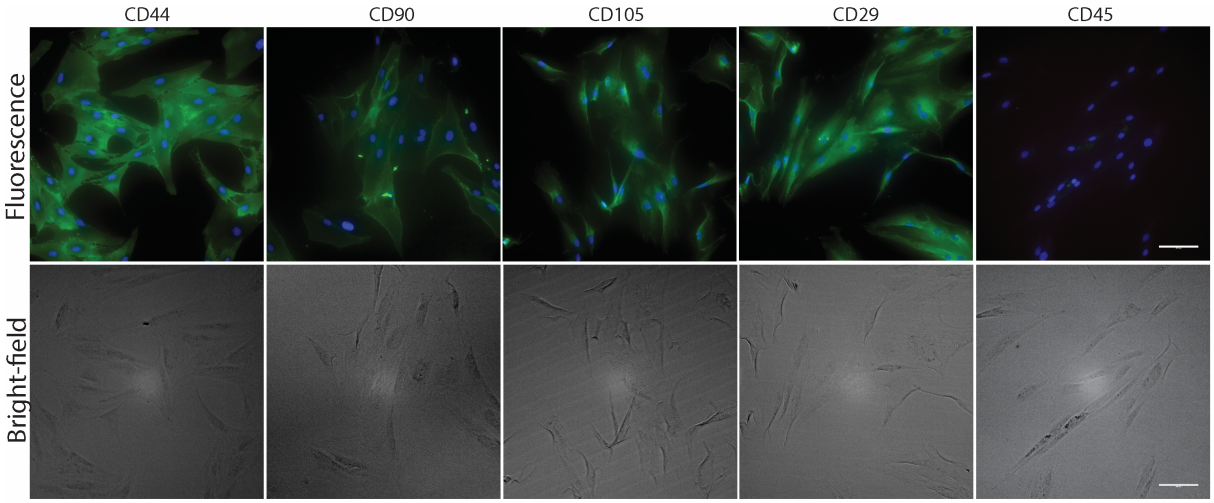

Figure S1: Characterization of human adipose derived stem cells by immunocytochemistry. ASC showed expression of the mesenchymal stem cell surface markers CD29, CD44, CD90 and CD105. CD45 is negative. Scale bar indicates 100 µm

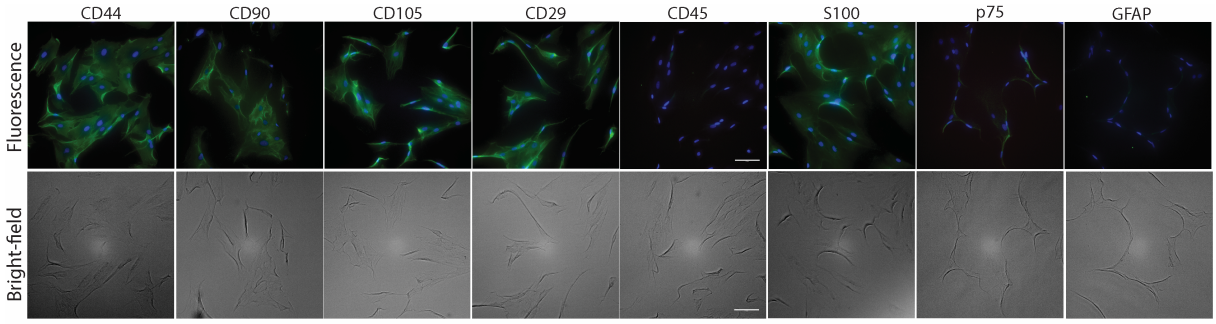

Figure S2: Characterization of NT3 stimulated human adipose derived stem cells by immunocytochemistry. ASC showed expression of the mesenchymal stem cell surface markers CD29, CD44, CD90 and CD105. CD45 is negative. Further, ASC showed expression of Schwann cell marker S100, but not p75 and GFAP. Scale bar indicates 100 µm

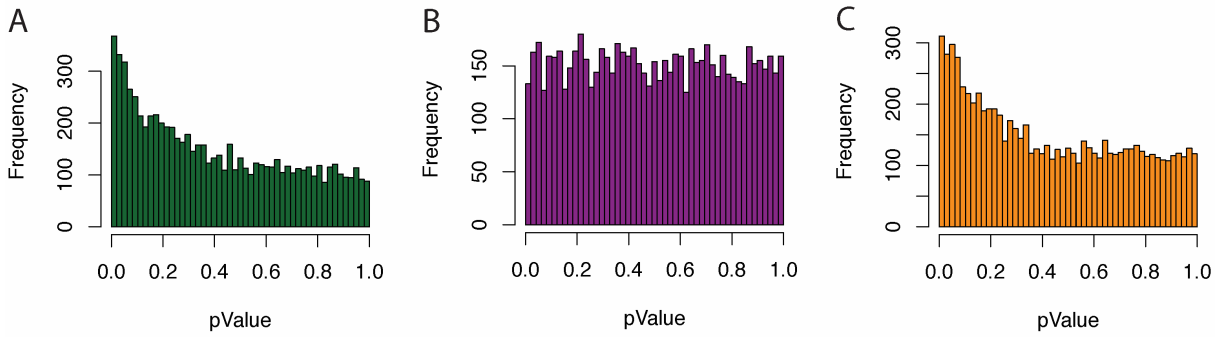

Figure S3: Frequency plots of p-values obtained for DRG explants with different treatment conditions compared to control GM. The plots for A) DRG treated with CM-NT3-ASC, B) DRG treated with CM- ASC, and C) DRG treated with NT3 alone are shown as generated by the SafeQuant script.

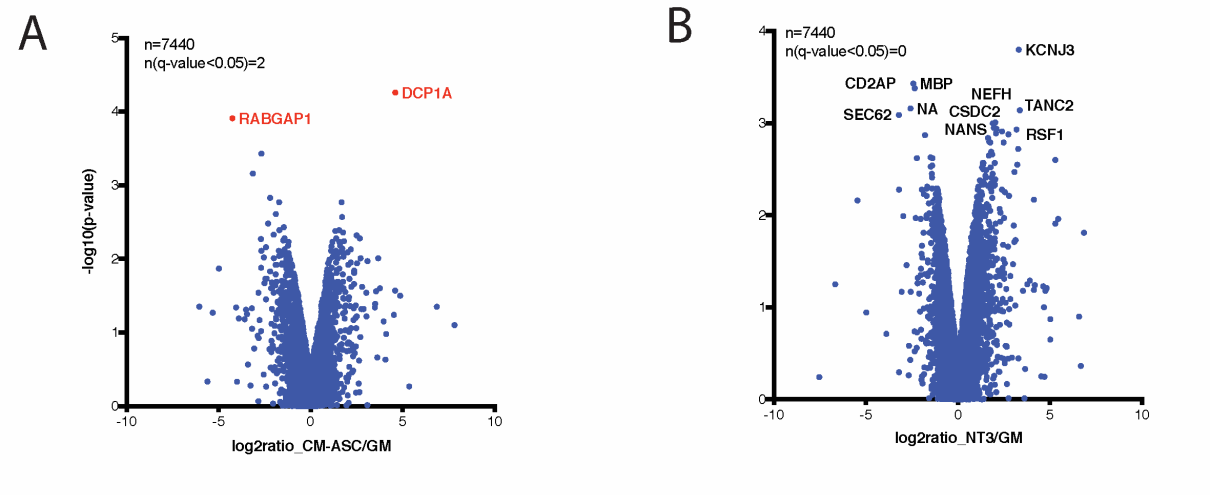

Figure S4: Quantitative phosphoproteomics analysis. A) Volcano plots of phosphorylation site ratios and significance (p-value) obtained for DRG treated with CM-ASC, and B) NT3. Significant hits with a q-value of less than 0.05 as computed by SafeQuant using paired t-test and Benjamini-Hochberg correction are to indicated in red, but no significance was found in comparison with control GM.

MANUSCRIPT 3

Optimization of Peripheral Nerve Reconstruction with Fibrin Sealant and Fibrin Nerve Conduits: A Prospective, Randomized Two-Arm-Controlled Non-Inferiority Phase-1 Clinical Trial

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Ongoing clinical study, to be finished by end of 2020

ABSTRACT

Introduction: Digital nerves are among the most frequently repaired peripheral nerves at hand surgery units worldwide. Even though standard treatment methods are well established, they are associated with several undeniable disadvantages that may have long-lasting impacts on the patients' quality of life. Thus, the aim of the present study was to investigate alternative treatment strategies involving fibrin gel for digital nerve injuries with no gap or a nerve gap-defect. **Methods:** In study 1, patients with non-gap digital nerve injuries were treated either with epineural suture (standard group 1a) or with epineural suture and fibrin sealant (treatment group 1b). In study 2, patients with critical sized (> 5mm) digital nerve gap injuries were treated either with an autologous nerve graft (standard group 2a) or with a fibrin nerve conduit (treatment group 2b). Follow-up outcomes evaluation was performed at 2 weeks and at 3, 6, and 12 months after surgery. For outcome analysis, data on adverse events, sensory recovery evaluated by static 2-point discrimination and Semmes Weinstein Monofilament test, visual analogue scale for pain, patient satisfaction surveyed by the Michigan Hand Outcomes Questionnaire for patient satisfaction, and nerve conduction by electroneurography, were acquired. **Results:** 36 patients with 46 nerve injuries were included in the study, with a mean age of 36 ± 14 years. No adverse events occurred. In study 1, group 1b experienced enhanced sensory recovery and higher patient satisfaction in the phase of early nerve regeneration compared to group 1a. In study 2, group 2b showed enhanced sensory recovery in comparison to group 1a, but lower patient satisfaction and higher pain sensation. Results, however, were not statistically significant. At 12 months' final follow-up assessment, standard and experimental treatments of study 1 and study 2 did not show any statistically significant differences in outcome parameters. Nerve conduction data were derived from all nerve injuries analyzed. **Conclusion:** Enhancement of early nerve regeneration by experimental treatment with fibrin sealant and fibrin conduit can be assumed, but would need to be confirmed by a larger patient population. The final outcome proves the non-inferiority of the experimental treatment options in study 1 and study 2 compared to the standard treatment. Fibrin conduits thus represent a viable alternative to autologous nerve grafts for the treatment of short-gap nerve injuries.

1. INTRODUCTION

The incidence (7–10 cases per 100,000 people) of nerve injuries of the hand and the wrist is rising, and the majority of injuries affect digital nerves [212,213]. Like all upper extremity nerve injuries, nerve injuries of wrist and hand are mainly caused by domestic or work-related accidents [4,5], and typically affect male and younger individuals (mean age around 30 years) [4–6]. Despite advances in microsurgical techniques, the functional outcome often remains suboptimal after the reconstruction due to reduced sensation, impaired motor function and in some cases pain [10]. Patients therefore experience a profound and sometimes permanent impact on their quality of life, and the economic implications for both, the patient and the society are considerable [4,5,12,13].

Sharp and clean nerve dissections with minimal tissue loss only are usually reconstructed by means of tension-free end-to-end coaptation using epineural suturing [30,214]. Primary repair and accurate coaptation of nerve endings with fascicular alignment and blood vessel continuity are likely to result in better outcomes and fewer complications for the patient [1,16]. However, epineural sutures generate inflammatory responses, which may result in increased fibrosis and thus inhibited axonal regeneration [31]. Thus, the concept of sutureless nerve repair has been studied [31]. Several studies investigated outcome and stability of nerve coaptations with the help of fibrin gel [32,33]. Reported results are conflicting. Some authors stated equivalent reliability and even superior outcomes of sutureless coaptions compared to epineural repair [34–36]. The advantages are obviously decreased inflammation and scar tissue formation. However, other authors reported on insufficient stability provided by the fibrin gel, which may lead to gapping or even disconnection of the nerve ends [37,38]. A valid compromise might be to perform a minimum of epineural sutures with stabilization of the suture site by fibrin gel. This technique resulted in increased tensile strength [39,40] and has been applied successfully in vivo [34,41], despite concerns that fibrin might impede the axonal regeneration [42].

In case of longer nerve defects, the nerve segments proximal and distal to the neurorrhaphy can usually not be sufficiently mobilized in order to perform a tensionless surgical repair at the site of injury [43]. Therefore, digital nerve gaps greater than 5 mm are commonly bridged with autologous nerve grafts taken from the patient's forearm or leg [44,45]. The nerve autograft provides a favorable environment for the outsprouting axons: an intact extracellular matrix for guidance and a population of residing Schwann cell

providing neurotrophic factors and adhesion molecules [1]. Nevertheless, the sacrifice of a healthy nerve is required, which not only creates a second surgery site, but also bears the risk of sensitivity loss, pain, and suboptimal scarring at the donor site. Furthermore, challenges are present at the recipient site as well, such as nerve size and type mismatch, neuroma formation, hypersensitivity, and only partial recovery of motor function [1,45]. Consequently, suitable non-autologous bridging options were researched, aiming at providing a growth supporting intraluminal microenvironment, and preventing aberrant axonal outgrowth and false reinnervation [47]. One promising alternative to autografts is currently seen in processed nerve allografts [44,54,215]. With the help of decellularization processes, any human leukocyte antigen (HLA)-target tissue is removed in order to avoid graft rejection and the need for immunosuppression of the patient [216]. Yet, the major challenge faced by allografts are ongoing improvements of these complex decellularization procedures [216]. Nerve conduits represent another intensively investigated treatment option. Nowadays, conduits used to guide and support outsprouting axons are made of biodegradable polymers, which can be produced from a large variety of synthetic or natural sources [45]. Among these, several FDA- and CE-approved "off-the-shelf" conduits are already commercially available for clinical use [55]. Clinical studies have been conducted on conduits constructed from collagen [217–220], Polyglycolic acid (PGA) [221–223] and Poly-ε-Caprolacton (PCL) [224].

Despite the variety of possible candidates, the optimal nerve conduit for clinical use has not yet been found. An optimal conduit would need to incorporate several crucial characteristics such as 1) easy handling and adaptability, 2) biocompatibility, 3) flexible consistency with good mechanical stability and tensile strength, 4) selective permeability for regeneration- and vascularization-associated factors, and 5) timely resorption after having supported the sprouting axons [11,57,225]. Fibrin is a natural protein within the coagulation cascade which has already proven to be indispensable in surgical practice. The clinical use of fibrin hydrogel as a hemostat, a sealant and an adhesive is FDA-approved [58–60]. Fibrin gel can be prepared autologously from individual donors, and it is also commercially available from different companies [32,58]. It has proven excellent biocompatibility in both cases [53,58,61]. Fibrin gel generally consists of two components: thrombin with calcium chloride, and fibrinogen with or without factor XIII and fibronectin, in some cases stabilized by an anti-fibrinolytic agent like aprotinin [61–63]. In order to adapt to its field of application, the

dissolving, coagulation, malleability and permeability characteristics of fibrin gel can be adapted by modifying the concentrations of its components accordingly [62,65,226]. A study of Moreno-Arotzena *et al.*[227] compared fibrin hydrogel with collagen hydrogel, where fibrin was shown to be associated with a lower void ratio, lower permeability and a significantly greater shear modulus. Added around the coaptation site of nerve ends, fibrin gel increased the tensile strength, which allows reducing the number of sutures [40,41]. *In vivo*, our group was able to show that fibrin nerve conduits (FNC) were capable of supporting short- and long-term axon regeneration [66–68]. In case of short gaps up to 10 mm, nerve regeneration with fibrin conduits even resulted in a similar extent of axonal sprouting and muscle recovery as with autografts [67,68].

Despite these highly promising outcomes for FNC *in vitro* and *in vivo*, the results have not yet been translated into humans. Therefore, this prospective, randomized two-arm pilot non-inferiority study was conducted in order to assess and compare the outcomes observed after experimental versus standard treatment of peripheral nerve injuries, either with no gap (study 1) or with a critical sized (> 5mm) defect (study 2). In study 1, the standard treatment comprised epineural sutures (group 1a), while the experimental treatment included additional fibrin sealant around the epineural sutures (group 1b). In study 2, the standard treatment involved an autograft (group 2a), while the experimental treatment used a fibrin nerve conduit (group 2b) to bridge the gap. We hypothesized non-inferiority for the experimental treatment options in terms of clinical outcome and patient satisfaction compared to the respective standard treatment. Validation of the experimental treatments for clinical use would allow optimized procedures of peripheral nerve repair in terms of depleted co-morbidities, reduced surgery time, implementation of easier techniques, and minimized costs.

2. MATERIALS AND METHODS

2.1. Study Design and Patient Population

This single-center two-arm-controlled pilot non-inferiority study was performed at the Department of Plastic, Reconstructive, Aesthetic Surgery, and Hand Surgery, at the University Hospital Basel. The study was in accordance with the principles announced in the Declaration of Helsinki, the guidelines of Good Clinical Practice (GCP) issued by the International Conference on Harmonisation of Technical Requirements for Registration of

Pharmaceuticals for Human Use (ICH), and Swiss regulatory authority's requirements. The study protocol was approved by the local ethics committee (Ref.Nr. EK: 241/12).

Patients aged between 18 and 90 years at the time of the surgery with a traumatic nerve injury of one or multiple finger(s) that was considered for peripheral nerve repair, were considered to be eligible for the study. Patients with known hypersensitivity or allergy to fibrin were excluded. After measuring the nerve gap during surgery, randomization envelopes were drawn either for study 1 or study 2, depending on the size of the nerve gap. In study 1, nerve repair for nerve defects < 5mm was performed. Patients were either randomized to group 1a (standard) and treated with epineural suture, or to group 1b (experimental) and treated with epineural suture and additional fibrin sealant. In study 2, nerve repair for critical sized (> 5mm) defects was performed. Patients were either randomized to group 2a (standard) and treated with autologous nerve transplantation from the lateral antebrachial cutaneous nerve (LACN) or to group 2b (experimental) and treated with a FNC. In cases where several fingers of one patient were affected, all fingers with a nerve injury without a gap were randomized to the same treatment group in study 1, and all fingers with a critical sized defect were randomized to the same treatment group in study 2. Since the study was designed as a pilot study we aimed at including 12 nerve injuries per group. The outcome of epineural suture with fibrin sealant (study 1) and FNC (study 2) were compared to the respective standard treatment option, i.e. epineural suture (study 1) and autologous nerve graft (study 2). Chart 1 provides an overview over the study design.

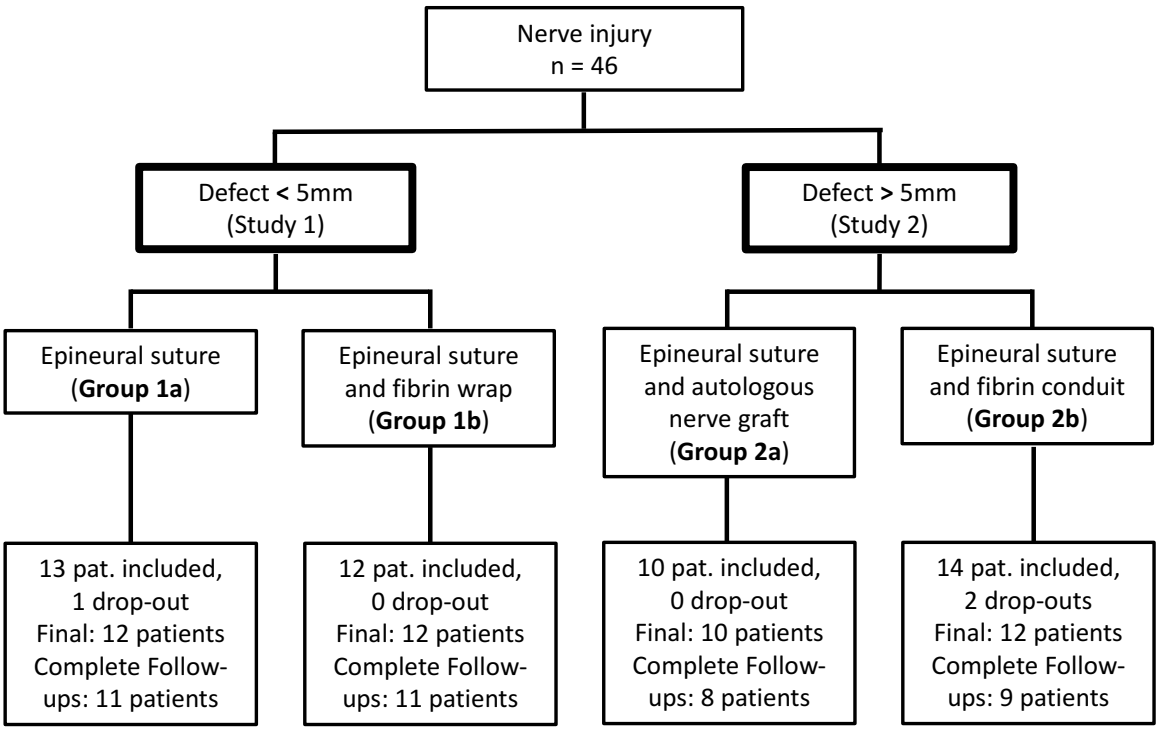

Chart 1. Study design, patients included, drop outs and completed follow-ups.

2.2. Fibrin Nerve Conduits

The FNC were freshly constructed during surgery using a two-compound fibrin hydrogel (Tisseel® Kit VH 1.0, Baxter SA, Switzerland). Tisseel provides a fibrinogen solution containing fibrinogen 72-110 mg/ml, factor XIII 0.6-10 IU/ml, plasminogen 40-120 mg/ml, aprotinin synthetic 3000 KIU/ml, and a thrombin solution 500 IU/ml with calcium chloride 40 µmol/ml. The fibrinogen and the thrombin were injected from two separate syringes through one head into the desired site where the fibrinogen polymerizes to fibrin. The fibrin gel was filled into a designed silicone inlay of a developed medical compressor around a stainless-steel core and pressed into shape using locking screws. The mold used was a silicone inlay (Dublisil 20®, Dreve-Dentamid GMBH, Unna, D), formed at the desired size of conduits. The inlay was surrounded by a cast (Jade-Stone®, Whip Mix Corp. Louisville, USA). Thus, uniform conduits of 14 mm length, 1 mm wall thickness, and 2 mm lumen were generated. After a 30 second compression using a 5 Newton compression screw, the conduits were harvested. They could easily be slipped from the stainless-steel core wire (50mm x 2mm), and resulted in stable conduits for implantation.

2.3. Surgical Treatment

All surgeries were performed by experienced microsurgeons. Damaged nerve ends were resected as needed. In the standard treatment group of study 1 (group 1a), the nerve ends were mobilized and adapted without creating tension at the coaptation site with two epineural sutures, which represents the minimum of sutures clinically acceptable for digital nerve repair in our experience. In the treatment group 1b, supplementary fibrin sealant was added by injecting fibrin gel around the suture site. In the standard treatment group of study 2 (group 2a), the LACN was transplanted as autograft. The proximal and distal nerve ends were each adapted with 2 epineural sutures. In the treatment group 2b, the proximal and distal nerve ends were inserted at each end for 2 mm into the fibrin conduit and attached to the conduit by a single epineural suture, creating a 10mm gap. Nerve gaps larger than 10 mm were not included into this study for reasons of comparability. Patients were subscribed to weekly occupational therapy sessions for up to 12 weeks, and, according to the specific clinical conditions, even longer.

2.4. Follow-up Procedures

Ambulatory follow-up assessments of patients were carried out at 2 weeks, 3, 6, and 12 months after peripheral nerve repair. At every clinical visit, we assessed scar quality and pain on a visual analogue scale (VAS), satisfaction scores according to the Michigan Hand Outcomes Questionnaire, surgical site infection according to CDC (Centers for Disease Control and Prevention), complication rate according to the Dindo-Clavien classification [228] and adverse events (AEs). At 3, 6, and 12 months after surgery, we additionally performed the static 2-Point Discrimination (s2PD) test, the Semmes Weinstein Monofilament (SWM) test, and nerve conduction studies.

2.5. Static 2-Point Discrimination (s2PD) Test

We used the touch test 2-point discriminator by Exacta (North Coast Medical, Inc., USA, Cat. No. NC12776). The discriminator was applied longitudinally to the finger at the palmar fingertip for at least three seconds in a random manner. The patient was asked to close the eyes and indicate perception by saying “one” or “two”, when perceiving one or two points, respectively. In case of an inaccurate response, the distance between the points was increased, in order to define the smallest detectable 2-point discrimination. The outcome

was expressed in millimeters (mm), and interpreted according to the modified American Society for Surgery of the Hand guidelines (modified ASSH) [229,230] (Table 1).

s2PD (in mm)	Score	Description
< 6	S4	Excellent
6 – 10	S3	Good
11 – 15	S2	Fair
> 15	S1	Poor or protective sensation

Table 1: Interpretation of the s2PD data according to the modified ASSH guidelines

2.6. Semmes Weinstein Monofilament (SWM) Test

We used a five-piece hand kit including monofilaments with target forces of 0.07g, 0.4g, 2g, 4g, and 300g (Aesthesio, DanMic Global, LCC, USA, Cat No. 514000-5C). The examination was performed over the entire palmar length of the respective digital nerve. The filament was applied at a force where it started to bend for a total of three times. The patient was asked to close the eyes and indicate perception of the touch by saying “yes”. The next higher monofilament strength was tested in case of missed perception in order to define the smallest detectable pressure at the fingertip of the impaired nerve. The results of the SWM test were interpreted according to the Imai-classification [229,231,232] (Table 2).

Monofilament strength (in gram)	Score	Description
0.07	S5	Normal tactile sensation
0.4	S4	Diminished light touch
2	S3	Diminished protective sensation
4	S2	Loss of protective sensation
300	S1	Basic deep pressure sensation

Table 2: Interpretation of cutaneous pressure threshold according to Imai

2.7. Visual Analogue Scale (VAS) for Pain

On a VAS ranging between 1 and 10, the patients were asked to indicate their perceived level of pain, score 1 meaning no pain and score 10 strong pain.

2.8. Michigan Hand Outcomes Questionnaire (MHQ)

In contrast to clinical outcome parameters, the MHQ questionnaire provides outcome scores based on the patients' perception of improvement or impairment of nerve and wound regeneration. The MHQ is a 37-item questionnaire covering the following six domains: overall hand function (OHF), activities of daily living (ADL), pain, work performance (WP), aesthetics and satisfaction with hand function. The scoring method was developed by Chung et al. [233]. Each item is scored on a scale between 1 and 5, and the sum for each category is converted into a scale ranging from 0 to 100. A high score indicates better hand function, except for the subscale "pain", where a high score refers to more pain. The domains OHF, ADL, aesthetics, and patient satisfaction contain questions for the right and left hand separately. The total MHQ score, which is composed of the average of all subscales, also ranges from 0 to 100, with a higher score indicating a better hand function. Reliability and validity of this questionnaire have been assessed in several studies [233–236]. In the present study, the German standardized translation was used [237].

2.9. Nerve Conduction Studies (NCS)

Sensory orthodromic nerve conduction studies (NCS) of the affected digital nerve were performed using surface electrodes. Sensory nerve conduction velocity (m/s) (NCV) and peak-to-peak amplitude of the sensory nerve action potential (μV) (SNAP) were assessed. Independently of the patient's age, normative values were defined for digital nerves derived from the median or the ulnar nerve based on the textbook published by Stöhr and Pfister [238]. A semi-quantitative scoring system was developed to assess the functional nerve outcome after surgery (Table 3).

	Median nerve	Ulnar nerve	Score
Normative values sensory NCV	≥47m/s	≥45m/s	1
Normative values peak-to-peak SNAP	≥7µV	≥6µV	1
Reduced values sensory NCV	<47m/s	<45m/s	2
Reduced value peak-to-peak SNAP	<7µV	<6µV	2
Not deducible	-	-	3

Table 3: Scoring of the electrophysiological measurements in nerve conduction studies

2.10. Cohort Size Calculation and Statistical Methods

Based on the non-inferiority hypothesis which predicts no statistical difference in clinical outcomes and patient satisfaction for the experimental treatment groups of studies 1 and 2 compared to their respective standard treatment group, 56 patients without neural gap (study 1) and 126 patients with a neural gap (study 2) are needed according to a provisional cohort size calculation. On this basis, we decided to perform a pilot-study first, which may be followed by a (multicenter) randomized-controlled trial with formal cohort size calculation.

Data were analyzed by unpaired Student's t-test and one-way analysis of variance (ANOVA) with Bonferroni correction for post hoc multiple comparisons, using SPSS (version 15.0; SPSS, Chicago, IL, USA). All tests were performed at a significance level of $p = 0.05$.

3. RESULTS

3.1. Population

For this manuscript data of 36 patients with 46 nerve injuries were analyzed. 12 nerve injuries each were included in groups 1a, 1b and 2b, while in group 2a only 10 nerve injuries could be examined up to now. 1 nerve injury in group 1a, 2 nerve injuries in group 2a and 3 nerve injuries in group 2b have not finished the study yet. After inclusion of another 2 patients for group 2a and completion of all follow-ups, a final evaluation of the complete data set will be accomplished for journal submission. Chart 1 provides an overview about the study design and progress.

64% of patients were male. The overall mean age was 36 ± 14 years (31 ± 12 years in group 1a, 43 ± 15 years in group 1b, 25 ± 7 years in group 2a and 38 ± 15 years in group 2b). The age distribution of the patient population is shown in Figure 1. Work-related nerve injuries

could be identified in 33% of the incidents. The reason for the injuries was related to knives in 36%, to glass in 22%, to drills or saws in 22%, and to other reasons in 19% of the cases. Only 11% of the patients were left-handed, and the dominant hand was affected in 36% of the injuries. Fingers involved in nerve damage where the thumb in 33%, the index finger in 28%, the middle finger in 11%, the ring finger in 7%, and the little finger in 22% of the cases.

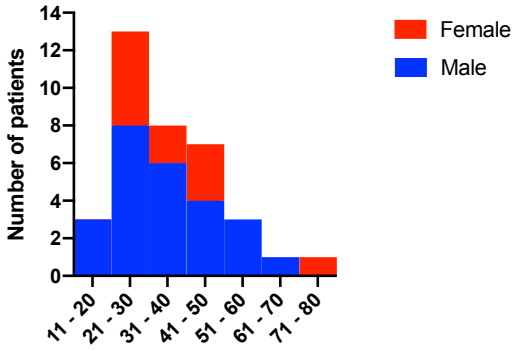

Figure 1. Age distribution of the studie's patient population at the time of the surgery. The bars represent the number of patients in a certain age category (in years).

3.2. Adverse Events

In group 2b, one polytrauma patient was included who developed hypertrophic scaring after 3 months, which improved during the follow-up. Also in group 2a, one patient experienced hypertrophic scarring due to wound manipulation, which also improved over time. No surgical complications, infections, or neuroma formation occurred otherwise.

3.3. Static 2-Point Discrimination (s2PD) Test

The s2PD measured at the healthy hand usually resulted in values of 3 – 6 mm (S4 or S3; data not shown). S3 at the non-affected hand was mainly obtained for patients with calloused skin. In study 1, no differences were found between the standard and the experimental group throughout the follow-up assessments (Figure 2 and Table 4). In study 2, group 2b showed enhanced sensory recovery at 3 and 6 months after surgery compared to group 2a (Figure 2 and Table 4). 12 months after surgery, the mean scores reached were 3.00 ± 0.63 for group 1a, 3.33 ± 0.49 for group 1b, 3.00 ± 0.76 for group 2a, and 3.22 ± 1.09 for group 2b. No statistically significant differences were found between the standard and

the experimental groups of studies 1 or 2. Details for the sensory recovery of each study group are shown in Supplementary fable 1 and Supplementary figure 1.

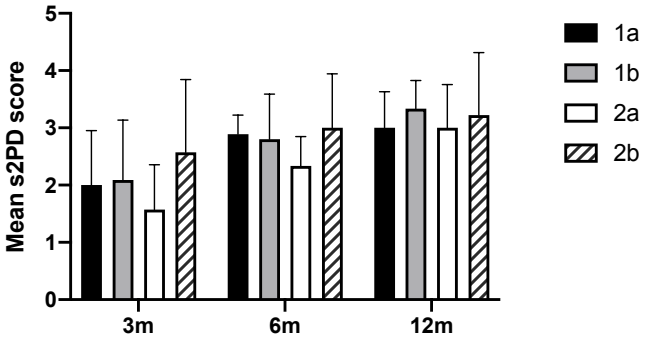

Figure 2. Data for the static 2-point discrimination (s2PD) test at 3, 6, and 12 months' (m) follow-up. The bars represent the mean ± SD of the score (ranging from S1 to S4) obtained by each study group. A high score is indicative for an improved functional outcome. No significant differences are observed.

	3 months	6 months	12 months
Group 1a	2.00 ± 0.95	2.89 ± 0.33	3.00 ± 0.63
Group 1b	2.09 ± 1.04	2.80 ± 0.79	3.33 ± 0.49
Group 2a	1.57 ± 0.79	2.33 ± 0.52	3.00 ± 0.76
Group 2b	2.57 ± 1.27	3.00 ± 0.94	3.22 ± 1.09

Table 4. Data for the static 2-Point Discrimination (s2PD) test at 3, 6, and 12 months' follow-up, expressed as the mean ± SD of the score (from S1 to S4) obtained by each study group.

3.4. Semmes Weinstein Monofilament (SWM) Test

Monofilaments tested at the healthy hand usually resulted in values of 0.07 – 0.4g (S4 or S3; data not shown). S3 at the non-affected hand was mainly obtained for patients with calloused skin. Evaluation of the pressure sensation revealed enhanced sensory recovery for the experimental groups 1b and 2b at 3 months after surgery compared to their respective standard treatment groups 1a and 2a (Figure 3 and Table 5). At 12 months, no statistically significant differences were found for study 1 between group 1a and group 1b with mean scores of 4.45 ± 0.52 for group 1a, and 4.33 ± 0.78 for group 1b. In study 2, experimental group 2b reached a significantly better sensory outcome compared to standard group 2a with mean scores of 3.63 ± 0.52 for group 2a, and 4.33 ± 0.71 for group 2b. Details for the sensory recovery of each study group are shown in Supplementary table 2 and Supplementary figure 1.

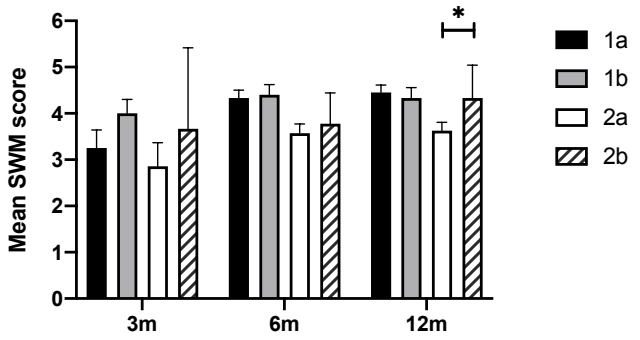

Figure 3. Data for the Semmes Weinstein Monofilament (SWM) test at 3, 6, and 12 months' (m) follow-up. The bars represent the mean ± SD of the score (ranging from S1 to S5) obtained by each study group. A high score is indicative for an improved functional outcome. Significant differences are indicated as P < 0.05*, P < 0.01** and P < 0.001***.

	3 months	6 months	12 months
Group 1a	3.25 ± 1.36	4.33 ± 0.50	4.45 ± 0.52
Group 1b	4.00 ± 1.00	4.40 ± 0.70	4.33 ± 0.78
Group 2a	2.86 ± 1.35	3.57 ± 0.53	3.63 ± 0.52
Group 2b	3.67 ± 1.75	3.78 ± 0.67	4.33 ± 0.71

Table 5. Data for the Semmes Weinstein Monofilament (SWM) test at 3, 6, and 12 months' follow-up, expressed as the mean ± SD of the score (ranging from S1 to S5) obtained by each study group.

3.5. Visual Analogue Scale (VAS) for Pain

In study 1, group 1a (score 1.92 ± 2.19) experienced more pain than group 1b (score 0.91 ± 1.51) at 3 months (Figure 4). For both groups, we observed a decline in pain 6 months after the surgery with most patients stating to be pain-free at that time (Table 6). In study 2, the evaluation revealed higher pain scores for group 2b compared to group 2a throughout all follow-up assessments (Table 6). Final scores at 12 months were 0.18 ± 0.60 for group 1a, 0.25 ± 0.62 for group 1b, 1.00 ± 1.69 for group 2a, and 1.89 ± 2.09 for group 2b. No statistically significant differences were found between the standard and the experimental groups of studies 1 or 2.

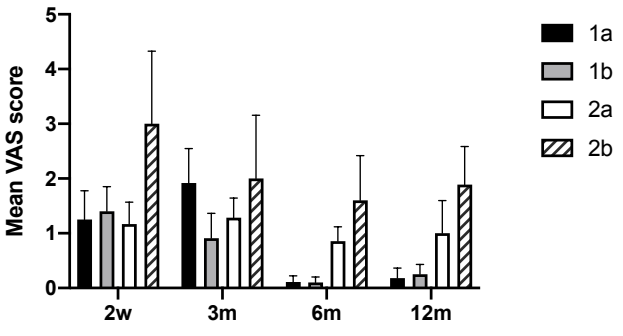

Figure 4. Data for the Visual analogue Scale (VAS) for pain assessment at 2 weeks' (w), 3, 6, and 12 months' (m) follow-up. The bars represent the mean ± SD of the score (ranging from 1 to 10) obtained by each study group. A high score is indicative for increased pain sensation. No significant differences are observed.

	2 weeks	3 months	6 months	12 months
Group 1a	1.25 ± 1.49	1.92 ± 2.19	0.11 ± 0.33	0.18 ± 0.60
Group 1b	1.40 ± 1.43	0.91 ± 1.51	0.10 ± 0.32	0.25 ± 0.62
Group 2a	1.17 ± 0.98	1.29 ± 0.95	0.86 ± 0.69	1.00 ± 1.69
Group 2b	3.00 ± 3.51	2.00 ± 3.06	1.60 ± 2.59	1.89 ± 2.09

Table 6. Data for the Visual Analogue Scale (VAS) score at 2 weeks', 3, 6, and 12 months' follow-up, expressed as the mean ± SD for the score (ranging from 1 to 10) obtained by each study group.

3.6. Michigan Hand Outcomes Questionnaire (MHQ)

The total MHQ score showed an important increase of the patients' overall satisfaction from the 2 weeks' to the 3 months' follow-up consultation for all study groups (Figure 5 and Table 7). In study 1, group 1b reached higher overall patient satisfaction than group 1a in the early nerve regeneration phase until 3 months after the surgery (Figure 5 and Table 7). In study 2, group 2a (82.0 ± 8.2) showed higher scores than group 2b (68.6 ± 24.2) at 3 months. For the follow-up assessments at 6 and 12 months, no significant score differences were found, neither between group 1a and group 1b, nor between group 2a and group 2b. Final evaluation after 12 months for the total MHQ score resulted in 90.4 ± 11.7 for group 1a, 95.7 ± 3.2 for group 1b, 85.7 ± 15.0 for group 2a, and 81.3 ± 13.9 for group 2b. The outcome for the subcategories are in line with the results displayed by the total MHQ, and are shown in Table 7.

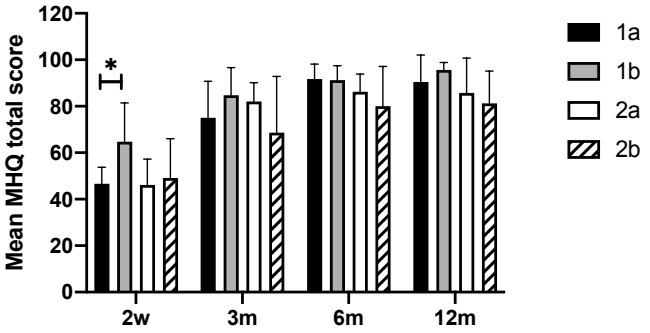

Figure 5. Data for the Michigan Hand outcome Questionnaire (MHQ) at 2 weeks' (w), 3, 6, and 12 months' (m) follow-up. The bars represent the mean ± SD of the score (ranging from 1 to 100) obtained by each study group. A high score is indicative for higher patient's satisfaction. Significant differences are indicated as $P < 0.05$*, $P < 0.01$** and $P < 0.001$***.

3.7. Nerve Conduction Studies

A statistically meaningful evaluation of the data was not possible due to a low patient engagement. Generally, we observed that sensory nerve conduction and conduction amplitude can be deduced for all tested patients after 12 months, in contrast to outcomes at 3 months or 6 months where NCV and SNAP for certain nerves could not be detected (Supplementary figure 2). Supplementary table 3 shows the measurements for each nerve injury. Patients generally seemed to experience an improvement of sensory nerve conduction velocity and amplitude over the follow-up period.

MHQ subcategories	Group	2 weeks	3 months	6 months	12 months
Total	1a	46.6 ± 7.1	75.1 ± 15.7	91.7 ± 6.4	90.4 ± 11.7
	1b	64.7 ± 16.7	84.8 ± 11.8	91.3 ± 6.2	95.7 ± 3.2
	2a	46.1 ± 11.1	82.0 ± 8.2	86.3 ± 7.5	85.7 ± 15.0
	2b	49.1 ± 16.9	68.6 ± 24.2	80.0 ± 17.1	81.3 ± 13.9
OHF	1a	35.0 ± 15.4	65.0 ± 23.7	81.7 ± 14.1	83.2 ± 15.0
	1b	57.5 ± 17.7	79.1 ± 17.9	86.5 ± 9.4	90.0 ± 7.7
	2a	40.0 ± 25.7	70.0 ± 16.6	78.6 ± 3.8	81.3 ± 16.2
	2b	50.0 ± 14.7	55.0 ± 21.4	70.6 ± 13.6	79.4 ± 15.1
ADL	1a	16.9 ± 23.3	84.4 ± 15.5	93.1 ± 8.4	89.0 ± 20.4
	1b	66.2 ± 23.5	88.9 ± 10.8	94.6 ± 5.9	96.7 ± 5.0
	2a	37.2 ± 23.0	90.2 ± 12.6	93.8 ± 4.3	91.7 ± 10.2
	2b	33.0 ± 18.3	69.9 ± 25.2	77.5 ± 17.6	79.2 ± 17.8
WP	1a	3.8 ± 5.2	57.5 ± 29.1	96.1 ± 4.9	88.6 ± 20.5
	1b	37.0 ± 33.0	76.8 ± 24.3	89.5 ± 14.6	98.8 ± 3.1
	2a	18.6 ± 24.8	87.9 ± 7.6	92.1 ± 9.1	85.0 ± 22.7
	2b	22.1 ± 33.1	62.1 ± 38.4	83.3 ± 25.6	82.2 ± 20.8
Pain	1a	29.4 ± 25.4	13.0 ± 17.0	1.1 ± 3.3	2.7 ± 9.0
	1b	23.0 ± 20.3	8.2 ± 13.5	4.5 ± 12.6	2.9 ± 7.5
	2a	32.9 ± 17.0	20.0 ± 14.1	11.4 ± 10.7	11.3 ± 20.3
	2b	33.6 ± 22.7	17.9 ± 20.2	15.6 ± 24.7	22.2 ± 17.7
Aesthetics	1a	95.3 ± 6.5	97.5 ± 6.0	97.2 ± 3.3	98.9 ± 2.5
	1b	89.4 ± 11.0	96.0 ± 4.2	93.8 ± 7.8	96.4 ± 7.8
	2a	68.8 ± 16.5	92.0 ± 4.7	93.8 ± 16.5	93.8 ± 17.7
	2b	79.5 ± 19.0	79.5 ± 28.8	87.5 ± 13.6	91.7 ± 18.2
Satisfaction	1a	58.3 ± 17.1	59.2 ± 29.2	83.3 ± 9.3	85.6 ± 13.6
	1b	61.3 ± 24.5	76.1 ± 21.2	87.9 ± 10.3	95.1 ± 5.0
	2a	45.2 ± 22.2	72.0 ± 13.3	85.1 ± 12.7	86.5 ± 15.1
	2b	43.5 ± 23.6	63.1 ± 24.6	76.9 ± 23.3	77.3 ± 13.5

Table 7: Data for the Michigan Hand Outcomes Questionnaire (MHQ) at 2 weeks', 3, 6, and 12 months' follow-up, expressed as the mean ± SD for the score (ranging from 1 to 100) obtained by each study group. Shown are the questionnaire's total scores and the subcategory scores; OHF = Overall Hand Function, ADL = Activities of Daily Living, WP = Work Performance.

4. DISCUSSION

In study 1, we compared outcomes of patients of the experimental treatment group 1b, in whom the coaptation site was additionally stabilized by fibrin sealant, with the outcomes of patients of the standard treatment group 1a, who received epineural sutures only. In the SWM test as well as concerning the answers of the MHQ, group 1b scored more favorably than group 1a until 3 months after surgery. In addition, pain perception on the VAS in group 1b was lower at 3 months. No difference in performance was seen for the s2PD. These results indicate an enhancement of early nerve regeneration in group 1b which might be attributed to the increased tensile strength at the coaptation site thanks to the fibrin sealant as reported earlier [39,40]. Assessments at 6 and 12 months resulted in similar outcomes for all parameters (s2PD, SWM test and MHQ) in both groups, and both groups reported a mostly complete pain relief from 6 months on. These outcomes imply equivalent nerve regeneration for standard and experimental treatment strategies for non-gap nerve injuries in the long term. Hereby, our results are line with other reports observing that fibrin glue does not inhibit the nerve regeneration, and is a suitable biomaterial to be used for peripheral nerve repairs [32,239,240]. Simultaneously, our data supports the statement of Mermans *et al*. [230], that follow-up is positively correlated with sensory outcome after digital nerve repair. According to these authors, a stable level of sensibility is reached by 6 months, which could be the time needed for functional reorganization. A multicenter study with formal cohort size calculation might allow to disclose significant differences in early functional nerve recovery between the standard and the experimental treatment group.

In study 2, we compared treatment outcomes of the experimental fibrin conduit group 2b with the standard nerve autograft group 2a. Sensory recovery in the experimental group 2b was enhanced at 3 months for the s2PD test as well as the SWM test compared to the standard group 2a, which implies improved early nerve regeneration for nerve repair with FNC. The outcome of group 2a balanced out in the s2PD test to the level of group 2b, but scores for pressure sensation tested with the SWM were significantly better in group 2b at 12 months after surgery. This is a novel finding, since nerve conduits tend to show an inferior regenerative potency compared to nerve autografts [232]. In contrast to clinical outcomes, pain reported scores were higher in group 2b compared to group 2a throughout the follow-ups, in line with a lower overall patient satisfaction in the MHQ for group 2b at 3 months after surgery. However, the VAS score might be biased due to undifferentiated

answer options. Patients usually reported unpleasant sensations such as tingling, burning, a buzzing “electric” feeling, or numbness more commonly than actual pain. Unfortunately, these sensations, which might be associated to incomplete nerve regeneration, were not documented in this study. Thus, the exact reasons why sensations of nerve irritation are slightly more frequent in group 2b remain to be investigated. Considering the statistical insignificance of the differences observed, non-inferiority of the experimental treatment with FNC compared to the standard autograft treatment for bridging 10 mm nerve gaps can be concluded. Moreover, nerve repair with fibrin conduit has several undeniable advantages: no sacrifice of a healthy nerve, no second surgery site, easier technique, and reduced surgery time and thus cost effectiveness. Therefore, nerve repair with FNC might not only present a viable alternative, but a new treatment strategy for short nerve gaps, as also suggested by Paprottka *et al.* [10].

The experimental treatments of both, study 1 and 2 showed tendencies towards resulting in a more favorable sensory outcome than standard treatments. Age has been discussed to strongly influence the sensory regeneration [10,230,241,242]. Since the mean age of the patients in both experimental groups was higher than the age of the patients in the two standard groups, this bias can be excluded. Nevertheless, the present study was created as pilot clinical trial, and the small number of patients per group (n = 12) may not allow a final statement on the true equivalence or differences between the treatment strategies. Moreover, the practice to allow the randomization and inclusion of the same patient for multiple nerve injuries in different fingers might have led to a bias in the functional, but also in the patients’ satisfaction outcome, assuming that the outcome largely depends on the patients’ compliance to therapy, expectations and resources. In addition, post-surgical care was not standardized, with patients following regular rehabilitation plans and patients not receiving rehabilitation at all. In study 2, only short nerve gaps up to 10 mm were evaluated for reasons of comparability. The performance of fibrin conduits in long nerve gaps could thus not be assessed. However, the purpose of this pilot clinical study was to provide preliminary data for a subsequent multicenter study with a formal cohort size calculation through which results of higher validity could be obtained. The introduction of the FNC into a clinical setting is a premiere and has yielded very promising outcomes. Moreover, this is the first clinical study evaluating an innovative nerve conduit which provides not only clinical functional outcomes, but also data on patient satisfaction, pain sensation, and on nerve

conduction. Compared to other equally promising bridging options in the field such as collagen conduits or the allograft, fibrin conduits present several advantages: with the appropriate molds, the surgeon can relatively spontaneously constitute fibrin gel conduits during the operation according to the respective requirements. Furthermore, fibrin gel is cost effective with a price of around $60/ml [58]. For constructing a fibrin conduit of 14 mm length less than one mm is needed.

Thus, additional fibrin sealant for non-gap-injuries and fibrin nerve conduits for gap-injuries represent a real alternative for the treatment of peripheral nerve injuries. The observation of enhanced early nerve regeneration remains to be validated, but the non-inferiority of the long-term outcome, as compared to standard treatments, was clearly shown. The ability of fibrin nerve conduits to also support long-gap nerve regeneration remains to be investigated. However, the optimization of empty fibrin conduits is an active field of research. Several interesting concepts have been developed, since a wide range of experimental fibrin applications exists in tissue engineering, Research around nerve regeneration specifically benefited from fibrin gel serving as delivery vehicle for drugs, neurotrophic factors, and stem cells [62,78,79,114,115].

5. CONCLUSION

Experimental treatment options, i.e the epineural suture combined with fibrin sealant and the fibrin nerve conduit, enhanced early sensory recovery and presented a non-inferior performance in the long-term compared to standard treatment options for no gap and 10 mm nerve gap injuries. This was observed by functional clinical outcome parameters and patient satisfaction levels at 12 months after surgery. A multicenter study with formal cohort size calculation would allow to draw a final conclusion on the equivalence or the possible superiority of the experimental treatment strategies involving fibrin gel compared to the standard treatments.

Authors contribution statement: D.K. supervised the research project; K.P., M.T., M.M.S. and B.D. conducted the study; K.P., M.T., M.M.S., D.K., D.S. and C.O. organized the database; K.P. and M.T. performed the statistical analysis; K.P. wrote the first draft of the manuscript; M.T., D.K. and C.O. revised the manuscript; All the authors contributed to manuscript revision, read and approved the manuscript submission.

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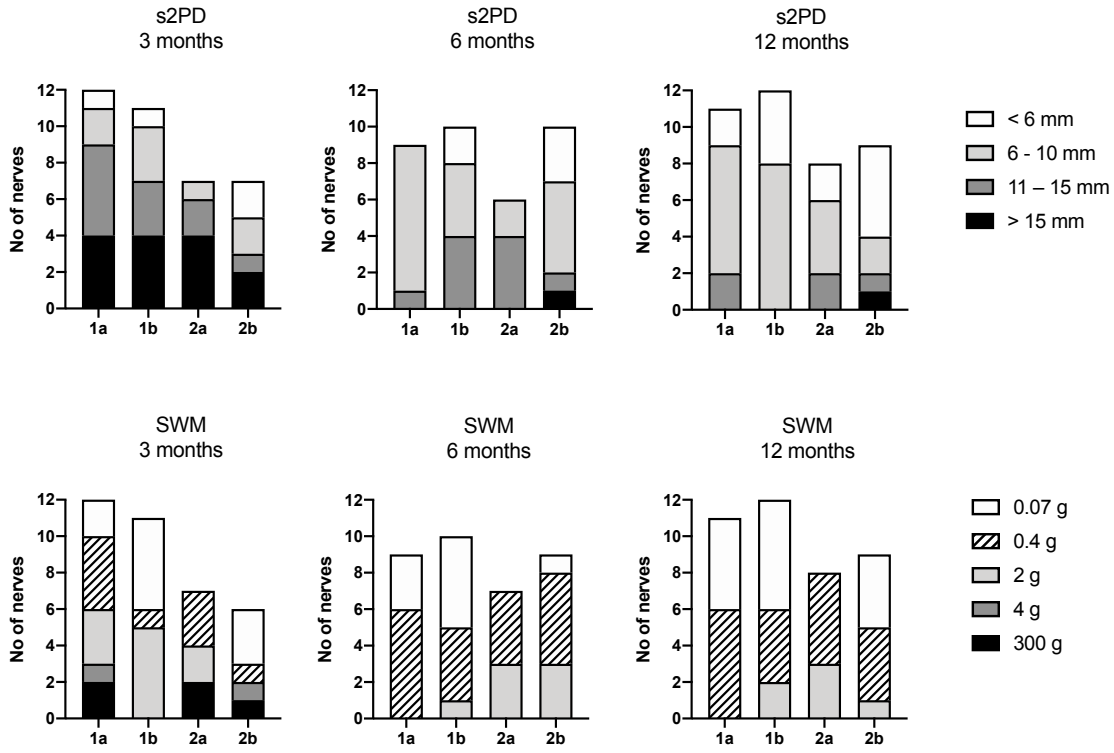
Disclosure: Authors declare no conflict of interest.

Supplementary figures and tables



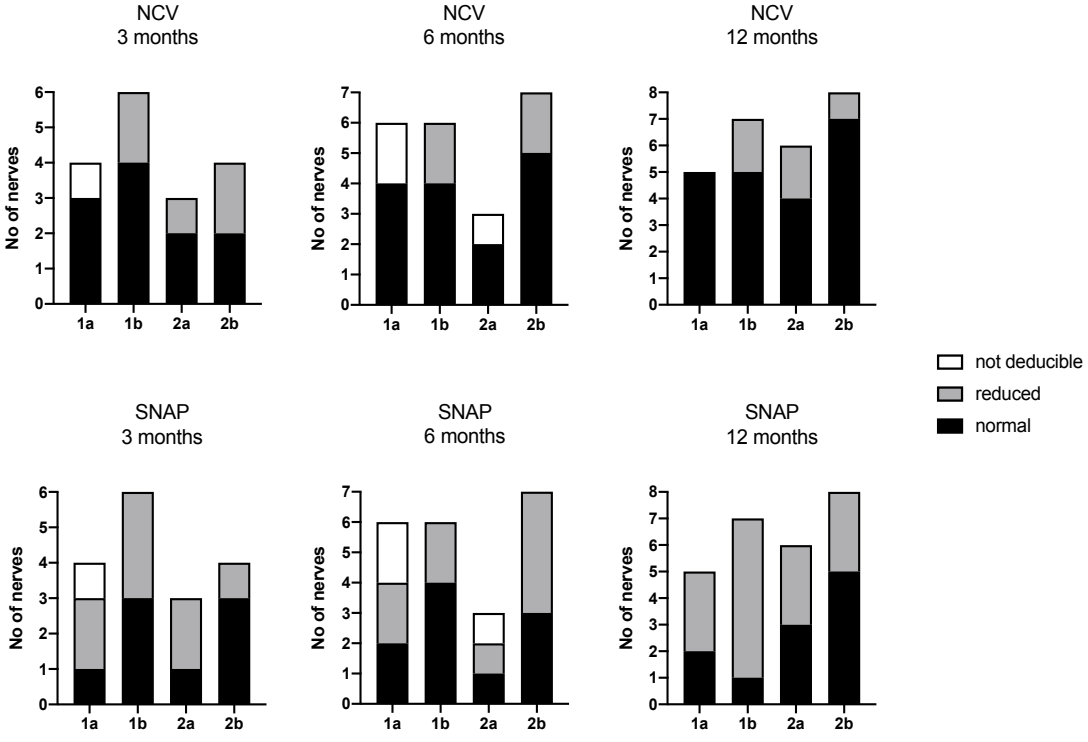
Supplementary figure 1. Data for the static 2-Point Discrimination (s2PD) test and the Semmes Weinstein Monofilament (SWM) test at 3, 6, and 12 months' follow-up. The bars represent the number of nerves showing the sensory recovery as labelled in the respective legend for each study group.

Time point	Group	S4: < 6 mm	S3: 6 – 10 mm	S2: 11 – 15 mm	S1: > 15 mm
3 months	**1a**	1	2	5	4
	1b	1	3	3	4
	2a	-	1	2	4
	2b	2	2	1	2
6 months	**1a**	-	8	1	-
	1b	2	4	4	-
	2a	-	2	4	-
	2b	3	5	1	1
12 months	**1a**	2	7	2	-
	1b	4	8	-	-
	2a	2	4	2	-
	2b	5	2	1	1

Supplementary table 1: Data for the static 2-Point Discrimination test at 3, 6, and 12 months' follow-up, expressed as the number of nerves showing return to sensibility on a score ranging from S1 to S4 for each study group.

Time point	Group	S5: 0.07 g	S4: 0.4 g	S3: 2 g	S2: 4 g	S1: 300 g
3 months	**1a**	2	4	3	1	2
	1b	5	1	5	-	-
	2a	-	3	2	-	2
	2b	3	1	-	1	1
6 months	**1a**	3	6	-	-	-
	1b	5	4	1	-	-
	2a	-	4	3	-	-
	2b	1	5	3	-	-
12 months	**1a**	5	6	-	-	-
	1b	6	4	2	-	-
	2a	-	5	3	-	-
	2b	4	4	1	-	-

Supplementary table 2: Data for the Semmes Weinstein Monofilament test at 3, 6, and 12 months' follow-up, expressed as the number of nerves showing return to sensibility on a score ranging from S1 to S5 for each study group.



Supplementary figure 2. Data for the electrophysiological measurements at 3, 6, and 12 months' follow-up for sensory nerve conduction velocity (NCV) and amplitude of the sensory nerve action potential (SNAP). The bars represent the number of nerves showing normal, reduced or not deducible values for each study group.

DISCUSSION AND OUTLOOK

The work completed during this MD-PhD thesis aimed at investigating innovative and translational treatment strategies for peripheral nerve repair and regeneration in different experimental settings. We focused on following three subjects: 1) new approaches in stem cell-based therapies by investigating the neurotrophic potency of ASC in response to specific growth factor stimuli, 2) the influence of the stem cell delivery route, i.e., FNC assisted intramural vs. intraluminal ASC-loading on early nerve regeneration, and 3) translation of the fibrin sealant and the fibrin nerve conduit techniques for peripheral nerve repair into a clinical context.

In the first manuscript, we hypothesized impactful change in the ability of ASC to support the axonal regeneration in response to growth factors' stimuli, i.e. NGF or VEGF. Thus, we first conducted an *in vitro* study where we analyzed the neurotrophic effect of NGF- or VEGF-stimulated ASC's conditioned medium (CM) on a chicken embryonic dorsal root ganglion (DRG) model. Subsequently, we translated the findings *in vivo* on a 10 mm sciatic nerve gap-model in rats, where we also studied two approaches of stem cell delivery, by either intramural or intraluminal ASC-loading of fibrin nerve conduits. In the second manuscript, we provided a comparative assay for the impact of six different NTF, i.e. NGF, BDNF, NT3, NT4, GDNF and CNTF, on the neurotrophic potency of ASC *in vitro* and studied the regenerative events taking place in the ASC and neuronal cells. In the third manuscript, we hypothesized non-inferior outcomes for two experimental nerve repair strategies, i.e. epineural suture with fibrin sealant or fibrin conduit, as compared to their respective gold standard treatment, i.e. epineural suture or autologous nerve graft, in a prospective, randomized two-arm- controlled *pilot clinical trial*. An additional two patients remain to be included to the study cohort, and the one-year follow-up of is still ongoing.

1. EX-VIVO NTF-STIMULATED ASC SHOW AN ENHANCED NEURO-REGENERATIVE POTENCY

Cell-based therapies for neuroregenerative purposes based on SC or SCLC are associated to several limitations, hindering their successful translation into a clinical context. Given the neurotrophic effect shown for ASC [104–106], and the fact that they express receptor binding sites for NTF [103,160–162] and are able to secrete a range of molecular growth signals [98,100], we hypothesized impactful change in the ability of ASC to support axonal

regeneration in response to growth factors' stimuli. Indeed, we were able to show an important enhancement of axonal regeneration *in vitro* and *in vivo* for experimental conditions involving NTF-stimulated ASC. Striking was also the consistent superiority in axonal outgrowth of NT3-stimulated ASC compared to other NTF. In line, we observed several significantly up-regulated regenerative mechanisms at a transcriptional and proteomic level in NT3-stimulated ASC and in DRG-explants cultured in the CM of NT3-stimulated ASC. These results give us reason to believe, that the receptor-bound signaling of exogenous NTF to ASC induces changes in their secretory behavior, which initiates important changes in the regenerative capacity of neuronal cells. However, in order to fully understand or prove this hypothesis, several additional experiments would need to be conducted. The question rises, how one can be sure, that the neurotrophic effect is not mainly to be attributed to the addition of exogenous NTF, since they exert a more potent effect in transcriptional and proteomic experiments than ASC alone.

Characterization of the changing secretome of stimulated ASC, the identification of the respective upregulated receptors on ASC and neuronal cells, as well as the assessment of the neuronal cell viability and survival after treatment would provide more insights into the above-mentioned topic. However, a clear enhancement of the neuroregenerative events taking place upon treatment with NT3-stimulated ASC compared to treatment with NT3 alone was demonstrated. These observations indicate the enhanced trophic potency of NT3-stimulated ASC. Furthermore, the distinct influence of different NTF on the axonal outgrowth from DRG-explants as shown in our in vitro assay might encourage further investigations on the joint impact of multiple NTF, since regenerative cells experience stimulation by numerous signals at the injury site [22,73]. Also a similar comparative assay using spinal cord explants might further improve the understanding of the distinct effects of NTF on different cell populations, since motor and sensory neurons react differently to axotomy, growth environment and treatment [243]. For our in vitro axonal outgrowth assay, co-culture with a trans-well system might have allowed further insides on the interaction between stimulated ASC and neuronal cells, such as the sustainability of the neurotrophic effect from stimulated ASC. However, this aspect is also demonstrated in our in vivo study where NGF-stimulated ASC enhance the nerve regeneration in rats. Taken together, ex vivo-stimulation of ASC by NTF as proposed in this thesis' results enhanced axonal outgrowth *in*

vitro and *in vivo*. NT3-stimulated ASC might be of special interest for future studies due to their extraordinary support of axonal regeneration.

2. IMPORTANCE OF THE CELL DELIVERY ROUTE FOR EARLY NERVE REGENERATION

An effective stem-cell based therapy also requires an adequate delivery of cells to the injury site. Fibrin gel provides a reliable scaffold where cells are able to adhere, spread and proliferate [112–114]. However, the most favorable stem cell delivery route in FNC has not been investigated yet. Thus, we evaluated two previously applied delivery techniques, i.e. intramural [116] versus intraluminal [115] ASC-loading of FNC *in vivo*. The results suggest that intramural delivery of NGF-stimulated ASC in FNC promoted better axonal regeneration than the respective intraluminal delivery. As discussed in manuscript 1, the differences in regenerative outcomes may be due to the variable density of transplanted cells within the hydrogel microenvironment, resulting in high concentrations of local growth factors. However, the events taking place after nerve injury involve up- but also down-regulation of certain growth factors during the different stages of nerve regeneration [22,73]. Moreover, the delivery of neurotrophic factors at high concentrations has been shown to be detrimental for axonal regeneration [74]. Thus, the highly enriched luminal microenvironment of the intraluminal delivery system may impede the speed of axonal regeneration in contrast to the intramural delivery system.

Despite improved neuro-regeneration by intramural delivery of NGF-stimulated ASC, the outcome of the autograft group remains superior, indicating a clear need for further refinement of this concept. It is not unlikely that transplanted NTF-stimulated ASC face the same fate as transplanted SCLC, which show a rapid reversion to stem cell-like characteristics after the withdrawal of the differentiation medium as shown by Faroni et al. [103]. As suggested by the authors of this paper, gene expression and protein level changes in NTF-stimulated ASC after a period of time would shed light on this issue. The question remains, how the enhanced regenerative properties of ASC can be maintained. Under the given experimental conditions however, intramural delivery showed enhanced early nerve regeneration independent of the cell type.

3. EXPERIMENTAL TREATMENT STRATEGIES FOR PERIPHERAL NERVE REPAIR WITH FIBRIN GEL ARE EQUIVALENT TO STANDARD TREATMENTS

Innovative and translational treatment alternatives for the surgical repair of peripheral nerve injuries are sought, in order to overcome the disadvantages of the current standard techniques [1,31]. Since fibrin hydrogel is an already widely used and accepted biomaterial in the surgical practice [58–60], its application for the peripheral nerve repair is of interest [32,63]. In order to avoid increased inflammation and fibrosis at the coaptation site of nerve ends, the approach of reduced number of epineural sutures with additional fibrin sealant for improved stability has been suggested [40,41]. Stability of the coaptation site might be of special importance in the early phase of nerve regeneration, where severed axons sprout and engage in path finding and reconnection with the distal nerve end. Indeed, the experimental treatment group 1b showed more favorable recovery of pressure sensation and overall patient satisfaction compared to the standard treatment group 1a until 3 months after surgery, indicating enhanced early nerve regeneration. Similar sensory outcomes and patient satisfaction, as well as nearly complete pain relief were observed for both groups at 6 and 12 months after surgery, which implies non-inferior nerve regeneration for the experimental treatment strategy as compared to the standard treatment for non-gap nerve injuries in the long term. Moreover, along with other authors, we were able to confound the worries about a possible detrimental impact of fibrin gel on the axonal regeneration [239,240].

In case of nerve gap-injuries, the FNC has been successfully reported to serve as a guiding structure *in vivo* [66–68] instead of the standard autologous nerve graft, with the advantage of depleted co-morbidities. Our results showed enhanced early sensory recovery for the experimental fibrin conduit group 2b compared to the standard autograft group 2a. Surprisingly, pressure sensation tested with the SWM reached significantly better scores in group 2b at the final 12 months' follow-up. Overall, non-inferiority for the nerve repair with fibrin conduits compared to the nerve autograft could be concluded due to otherwise statistically insignificant differences in treatment outcomes. A (multicentre) study with formal cohort size calculation would allow to support our findings made for study 1 and study 2.

There exist several limitations in the design and the conduct of the study as discussed in manuscript 3, which might have caused a bias of the findings of this study. Additionally,

several examiners were involved in the study, which might have generated an inconsistency in examination techniques of clinical outcomes. Also the time points of patient follow-ups were rather heterogeneous, even though patients were asked to return for ambulatory assessments. However, in strong favor for our clinical study, it provides a wide range of outcome parameters, i.e. clinical outcomes, patient satisfaction and nerve conduction studies, which is rarely found in other comparable studies. This allows a more holistic view on the treatment outcomes. Since only short nerve gaps of 10 mm length were investigated, the performance of fibrin conduits for supporting longer nerve gap injuries remains to be investigated. In order to enhance the supportive capacity of fibrin nerve conduits also for long-gap nerve injuries, various improvements via stem cell-, NTF- or drug-delivery [62,78,79,114,115] are being investigated.

CONCLUSION

This MD-PhD thesis investigated innovative and translational treatment options for peripheral nerve regeneration and repair. ASC showed a significantly improved neurotrophic potency upon stimulation with various NTF, in particular in response to NT3. Upregulation of regeneration-associated pathways were detected in ASC as well as in sensory neurons. In vivo, intramural delivery of NGF-stimulated ASC in fibrin nerve conduits offered improved support for the early nerve regeneration in comparison to intraluminal delivery. Together, these findings provide new knowledge and possibilities for the development of ASC-based therapies. Moreover, a direct comparative overview over the neurotrophic effect of various NTF is given. In the clinical context, treatment of patients for digital nerve injuries with epineural suture and fibrin sealant or fibrin conduit resulted in improved early nerve regeneration. The experimental treatment options performed non-inferior to the standard treatment options in the long-term outcome and might therefore present viable alternatives for the treatment of non-gap and short-gap nerve injuries.

CHALLENGES AND PERSPECTIVES FOR FUTURE RESEARCH

The topic of peripheral nerve regeneration and repair comprises many domains of research, and this MD-PhD thesis has offered new insights on how an improved functional outcome for patients might be obtained. Nevertheless, our findings also created a strong basis for further scientific questions. How sustainable is the ex-vivo stimulation of adipose stem cells,

specifically for in vivo usage? What are the exact molecules contained in the secretome of stimulated ASC that cause the enhanced neuro-regeneration? What combination of NTF would provide even stronger improvements of the regenerative capacity of ASC? Are fibrin nerve conduits also applicable for long-gap nerve injuries? And of course, is a translation of stem cell-based therapies for the improvement of nerve conduits into clinical use possible? These are critical questions, but the suboptimal outcome for patients with peripheral nerve injuries treated with conventional treatment strategies has made clear, that nerve regeneration requires more than just a perfect microsurgical approach. Co-morbidities of the therapy and also the maintenance of an active regenerative microenvironment are demanded to be addressed.

CLOSING REMARKS

I had the great honor to be part of the research team of Prof. Daniel Kalbermatten during my MD-PhD studies in translational research. I was able to perform *in vitro* projects based on tissue from different species. For *in vivo* studies, I learned the correct handling of rodents and investigated the manufacture of an optimized fibrin conduit as well as techniques of optimized stem cell loading. I was also involved in a *phase-1 clinical study*, which provided me insights and knowledge in clinical research. I was able to elaborate own ideas and to plan and conduct studies from the very beginning on under the excellent supervision by PD Dr. Srinivas Madduri and inputs of my colleagues. I learned a tremendous amount about the use of various methodological strategies in order to demonstrate and validate my findings. Not only was I constantly encouraged to broaden my horizon through a continuous literature review, but also the classes I attended at the university, as well as the contact and the discussions with the researchers in my environment shaped and sharpened my scientific understanding. Thus, I am proud to finish my PhD with three manuscripts, which include one published paper, one submitted manuscript, and one manuscript in the pipeline for submission.

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CURRICULUM VITAE

Personal data

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Academic education

Oct. 2016 – April 2020	MD-PhD degree, research group of Prof. D. Kalbermatten, University of Basel
2013- 2016	Master`s degree in medicine and Swiss Federal Medical Examination, University of Basel
2010- 2013	Bilingual Bachelor`s degree in medicine (French and German), Université de Fribourg
2006- 2010	Bilingual high school graduation (English and German), Alte Kantonsschule Aarau

Medical traineeships

April - Dec 2015	Medical electives in Switzerland (surgery, radiology, anesthesia, internal medicine) and Melbourne, Australia (plastic surgery)
Sep 2014	Summer school for disaster medicine and humanitarian aid, Ulm (Germany)
Sep 2013- March 2014	Weekly practice at a family doctor`s office, Rupperswil
July 2012	practical training in nursing at the Hôpital cantonal de Fribourg

Institutional responsibilities (selected)

June 2018- ongoing	Actionuni representative for SNF councils
April 2018- Jan 2020	Member of the Working Group “Graduate School Health Sciences” at the University of Basel
Mar 2018- ongoing	Member of the Senate Committee for Equal Opportunity at the University of Basel
Oct 2017	Committee member and organizer of the 6th European MD-PhD Conference, October 13-14th 2017, Basel
Dec 2015- Jan 2017	Member of the Medical Faculty Assembly at the University of Basel
2010- 2013	Active member and anatomy tutor at the student association of the Faculty of Medicine at the University of Fribourg

Supervision of students

Dec 2019- ongoing	Matura student: Anna Schären

Work experience (selected)

June 2014- Oct 2016	Night watch for inpatients at the Cantonal Hospital of Aarau
July 2013	Nursing assistance at the Hospital of Menziken
2005- 2007	Columns author at Aargauer Zeitung

Memberships

Swiss Society for Neuroscience
European and Swiss MD-PhD association (EMPA and SMPA)
Actionuni der Schweizer Mittelbau
Avuba- Assistierendenvereinigung der Universität Basel

Awards, Grants and Certificates

2019	Doctoral scholarship grant of the Goldschmidt-Jacobson-Stiftung
2018	Microsurgical Basic Course, DAM Certification
2007	3. rank at the „Swiss Youth Music Competition“, category piano
2005	1. rank at the Hans Christian Andersen Literaturwettbewerb 05, Baden
2005	3. rank at the „Swiss Youth Music Competition“, category piano
2003	Participation at the „Swiss Youth Music Competition“, category piano

Talks and poster presentations (selected)

23. Mai 2019	30th EURAPS Annual Meeting, Helsinki, Finland (Talk)
22. Mai 2019	8th EURAPS Research Council Meeting, Helsinki, Finland (Talk) **Best Paper award**
08. Nov. 2018	40. Jahrestagung der DAM
21th Sep 2018	54. Jahrestagung Swiss Plastic Surgery, Crans Montana, CH (Talk)
16th May 2018	29th EURAPS Annual Meeting, Madrid, Spain (Talk)
12th Oct 2017	3rd Basle-Manchester Forum (BMF), Basel, CH (Poster)
14th Oct 2017	6th Swiss and European MD-PhD Conference, Basel, CH (Talk)
01th Sep 2017	53. Jahrestagung Swiss Plastic Surgery, St. Moritz, CH (Talk)

Professional stays abroad

Nov- Dec 2015	Medical elective, Plastic surgery, Sunshine hospital (Melbourne, Australia), Head of department: Mr Wai-Ting Choi

Languages

German:	First language
English:	Bilingual graduation Cambridge Certificate of Proficiency in English (CPE) (C2)
French:	DELF C1